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Although the proteolysis of synaptobrevin by botulinum neurotoxin (BoNT) type B has been well characterised, much less is known about the efficient cleavage of SNAP-25 by BoNT/A and /E. Therefore, an ELISA was optimised and standardised for measuring their proteolysis of immobilised, bacterially-expressed SNAP-25, using purified IgGs reactive solely with full-length or BoNT/A-truncated SNAP-25. Polystyrene-attached SNAP-25 proved an excellent substrate for BoNT/A relative to the soluble or native protein. A small SNAP-25 C-terminal peptide (residues 181-206) was cleaved ~200-fold less efficiently than the intact molecule, establishing that sequences distant from the scissile bond are a prerequisite for optimal activity. P2, 1, 1' or 2' residues in SNAP-25 were altered by site-directed mutagenesis (one- and two-step PCR procedures or the efficient *Dpn*-1 nuclease-quick-change method). Glutathione-S-transferase-linked SNAP-25 variants - incorporating single, double or triple mutations, addition or deletions at the C- and N- termini - have been isolated by affinity chromatography. Their susceptibilities to proteolysis by BoNT/A are currently being examined to reveal residues distant from, and adjacent to, the cleavage site that are needed for the toxin's efficient action. Eventually, both these domains will be incorporated into a 'down-sized' ideal substrate before its modification to make an effective inhibitor. An additional strategy will entail creation of a toxin-resistant SNAP-25 variant that is still able to support transmitter release and, thus, have the potential to rescue botulinised nerve endings when targetted with an available cholinergic transporter.

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Professor J.D. Dolly 18th Sept 98

PI - Signature Date

TABLE OF CONTENTS

THE ACCESSION DOCUMENT (AD) **1**

REPORT DOCUMENTATION PAGE **2**

FOREWORD **3**

INTRODUCTION **5**

MATERIALS AND METHODS **6**

RESULTS AND DISCUSSION **13**

CONCLUSIONS **24**

REFERENCES **25**

APPENDIX **27**

TABLE 1 27

FIGURE LEGENDS 28

FIGURES 34

INTRODUCTION

Elucidating the molecular basis for the remarkable substrate selectivities of the zinc-dependent protease activities of botulinum neurotoxins (BoNTs) and tetanus toxin (TeTx), which target single peptide bonds of sole substrates (except BoNT/C which cleaves two substrates; Foran *et al.*, 1996; Williamson *et al.*, 1996), is essential for the design of useful high-affinity inhibitors. Towards this end, many researchers have developed quantitative reverse-phase HPLC assays for assessing cleavage of synthetic synaptobrevin (Sbr) peptide homologues (ranging from 34-62 residues in length) by BoNT /B, /D, /F, /G and TeTx (Shone *et al.*, 1993; Foran *et al.*, 1994; Cornille *et al.*, 1994, 1997; Wictome *et al.*, 1996; Soleilhac *et al.*, 1996; Pellizarri *et al.*, 1997). Sbr residues positioned on either side of the BoNT/B cleavage site were found to be equally essential for defining an efficient substrate (Shone and Roberts, 1994). It has been proposed that the large number of extra residues required for optimal activity allow a particular folding of the susceptible polypeptide, or provide extra domains for interaction with the toxin's light chain (LC). In the case of TeTx, compelling evidence has been presented for distant regions inducing conformational changes in the LC converting it into an active form, thereby, accounting for the high degree of substrate selectivity (Cornille *et al.*, 1997). For BoNT/A, Schmidt and Bostian (1995, 1997) found that a 17-amino acid peptide corresponding to residues 187-203 of SNAP-25 could serve as a substrate; extending its length to 66 residues gave much better cleavage (Washborne *et al.*, 1997). Thus, it appears that the 9 residue SNARE motif (145-153), located far upstream of the scissile bond of type A (Rossetto *et al.*, 1994), is essential for efficient recognition by the toxin.

As a prelude to our prime goal of designing an effective substrate-based inhibitor of BoNT/A, and considering the need to incorporate exo-site residues onto the minimal size proven substrate, identification of such important amino acids in full-length, recombinant SNAP-25 was sought prior to its 'down-sizing'. This offered the advantage of exploiting rapid PCR mutagenesis and protein expression/purification technologies to allow investigation of numerous SNAP-25 mutants. For ease of analysis of the toxin susceptibilities of these multiple mutated proteins, a rapid and sensitive ELISA (Hallis *et al., 1996;* Ekong *et al.*, 1997) was adapted for quantitation of BoNT/A-induced proteolysis. This relied on the preparation of purified antibodies reactive solely with intact or BoNT/A-truncated SNAP-25; these facilitated quantitation of the cleavage of the substrate or appearance of product by

19981029 027

enzyme-linked colourimetric development.

In order to identify residues of SNAP-25 which contribute to its interaction with the active site of BoNT LC, amino acids at the P1, P1`, P2 or P2` positions [nomenclature of Schechter and Berger (1967) where the P1 and P1` residues lie on the N or C-termini of the scissile bond, respectively] have been altered by site-directed mutagenesis. A large number of single, double and triple mutants plus variants with addition or deletions of residues at C- and N-termini have been expressed as glutathione (GST)-linked fusions and purified; their proteolytic susceptibilities to BoNT/A and /E are now being investigated. Whilst some of the SNAP-25 mutations complement studies performed by Schmidt and Bostian (1997) where changes were made to a C-terminal 17-mer (187-203) synthetic SNAP-25 substrate, the additional mutants prepared herein permit new facets to be addressed, especially multiple substitutions and size reduction by N-terminal truncations with, importantly, retention of the SNARE motif.

MATERIALS AND METHODS

Materials. Highly purified BoNT/A and /E were supplied by Drs Gary Lawrence (Imperial College, London) and Bibhuti DasGupta (Madison, Wisconsin). An antiserum specifically recognising the N-terminal product resulting from BoNT/A-mediated cleavage of SNAP-25 was a gift from Dr Theresa Ekong (NIBSC, Herts, UK.). Antibody preparations reactive with C-terminus of SNAP-25 were raised against a synthetic peptide encompassing residues 195-206 and affinity-purified on the immobilised immunogen. Mouse cDNA clones (GST SNAP-25_{FL}) containing the entire coding region of SNAP-25b (206 residues) and GST SNAP-25_A, a BoNT/A truncated SNAP-25 (1-197 residues) were provided by this laboratory. ELISA plates (Probind or Microtest III) were purchased from Becton Dickenson (Crawley, Oxford). Fraction V bovine serum albumin (BSA) was from ICN Flow Ltd. Immobilon-P membrane was from Millipore. Enhanced chemilumenescent substrates were purchased from Amersham International (Amersham, U.K.). All the other reagents used were of the highest purity available and supplied by Sigma Chem Co Ltd (Poole, Dorset) including BSA, the tris salt of *p*-nitrophenyl-phosphate (pNPP), anti-guinea pig or anti-rabbit antibodies conjugated to horseradish peroxidase or alkaline phosphatase. The suppliers of other specialised products are detailed in the methods.

Expression of GST SNAP-25 in *E. coli.* GST-SNAP-25 and GST-SNAP-25_A were

expressed using methods described by Smith and Johnson (1988). Bacteria containing the pGEX-2T plasmid incorporating the appropriate SNAP-25 gene were grown overnight at 37°C with vigorous shaking in 5 ml of Lauri-Bertani (LB)-medium containing 100 μg/ml ampicillin and diluted 200-fold into 1 L of the same medium. This mixture was incubated at 37 °C with vigorous agitation until an optical density of 0.6 units at 600 nm was reached; at this stage, isopropyl-β-D-thiogalactopyranoside (IPGT) was added to 0.4 mM concentration and the incubation continued for 4 h. The bacteria were harvested by pelleting at 4,000 g (max) for 10 min; this and all subsequent procedures were performed at 4°C. The resultant bacterial pellet was resuspended in 10 ml Tris-buffered-saline [TBS; consisting of 25 mM Tris.HCl, pH 7.5 containing 0.15 M NaCl) supplemented with 2 mM EDTA, 0.5 mM PMSF, 0.5 mM benzamidine, 1 mM DTT, 5% glycerol, 1 mg/ml lysozyme and one of the following detergents: 1% (w/v) Lubrol-PX (also known as Thesit) or Triton X-100. Bacteria were then lysed by brief sonication whilst on ice; insoluble material was removed by ultracentrifugation at 200, 000 g (max) for 30 min. The resultant supernatant was retained and subjected to affinity chromatography.

Purification of GST SNAP-25 from bacterial lysates using affinity chromatography. The resultant high-speed supernatant was added to 2 ml of glutathione-Sepharose and the mixture incubated batch-wise by gentle rotary shaking at 4°C for 1 h. The mixture was added to a 10 ml column and the resultant breakthrough put through at least 3 times to maximise binding. Next, the resin was washed using ten 5 ml aliquots of 2 x strength TBS buffer to remove proteins interacting non-specifically. Finally, the column was equilibrated with 5 ml of TBS, prior to bio-specific elution of the fusion proteins with 10 x 1 ml aliquots of TBS buffer (pH 8.0) containing 10 mM glutathione and 10% (v/v) glycerol. The protein content was determined using a colourimetric method (Bradford, 1976) and the pooled material frozen at -60°C as aliquots. When necessary, purified recombinant GST-fusion proteins was incubated with thrombin (purified from human plasma; activity ~ 200 units/mg protein; Sigma Chem Co. Ltd.) in TBS buffer, pH 7.5 for 2 h at 37°C at 1:100 (w/w) enzyme:substrate ratio to cleave the susceptible bond located between GST and SNAP-25

Coating GST-SNAP-25 onto ELISA plates. Polystyrene 96-well plates were incubated with GST-SNAP-25 (50 μl of 2 μg/ml per well) in a modified KGEP buffer [consisting of: 20 mM piperazine-N-N`-bis(2-ethanesulphonic acid, PIPES).NaOH, pH 7.0, 140 mM potassium glutamate, 5 mM $MgCl_2$, 0.5mM EGTA, 50 μM $ZnCl_2$ and 0.02% (w/v) NaN_3] for a

minimum of 1 h at 23°C. Unbound substrate was removed by brief rinsing with 2 x 0.15 ml aliquots of TBS buffer. The wells were blocked for a minimum of 1 h at 23°C, or overnight at 4°C, by incubation with 0.15 ml aliquots of TBS containing 2% (w/v) BSA (fraction V) and 0.02% (w/v) NaN_3.

Exposure of ELISA plates coated with GST-SNAP-25 to pre-reduced BoNT/A. Prior to use, blocking buffer was aspirated and wells washed with 2 x 0.15 ml TBS buffer. BoNT/A (maximum conc. 1 μM; otherwise, rapid precipitation may occur) was pre-reduced in the presence of 20 mM DTT for a minimum of 45 min at 37°C; variable concentrations of BoNT/A were prepared in KGEP buffer containing 0.2 mg/ml BSA as a carrier protein and 1 mM DTT. Where specified in the Figure legends, reduced BoNT/A was pre-incubated for the specified periods with different test reagents. After adding 75 μl of the toxin solutions, the plates were incubated at 37 °C for variable periods; the wells were then aspirated and washed with 2 x 0.15 ml of TBS containing 0.2 mM EDTA to inhibit residual protease activity (if present). After aspiration, 75-80 μl of primary Ig in blocking buffer containing 0.2 mM EDTA and the appropriate anti-SNAP-25 Ig (at the concentrations specified in Figure legends) was added and incubated typically for 16 h at 4°C or for 2 h at 23°C. Unbound IgG was removed by aspiration with 4 brief washes with 0.15 ml TBS (performed within 20 min per plate). Plates were then incubated with 1:1000 (v/v) dilution of anti-species IgG (rabbit or guinea pig) conjugated to alkaline phosphatase in blocking buffer for 2 h at 23°C. After removal of all the latter medium, 100 μl of alkaline phosphatase development buffer (0.2M Tris.HCl, pH 9.5, 0.1 M NaCl, 1 mM $MgCl_2$ and 10 μM $ZnSO_4$) containing 3.5 mM pNPP substrate was added, and the colour development allowed to proceed. The reaction was terminated by the addition of EDTA to a 10 mM final concentration and absorbancies at 405 nm measured using an ELISA plate reader.

Covalent-labelling of GST SNAP-25 or a C-terminal peptide with biotin. Affinity-purified GST SNAP-25 was subjected to size exclusion HPLC on a Waters-Millipore 300SW column (dimensions 30 cm x 0.78 cm^2) pre-equilibrated with 0.2 M $NaHCO_3$ (pH 8.4) in order to remove glutathione present (remaining after purification). The appropriate peak fractions were collected and concentrated using a Centriprep-30 device (obtained from Amicon Inc). Biotin-BMCC (purchased from Pierce and Warriner Ltd, Chester U.K) was added to GST SNAP-25 (0.5 mg protein/ml) in 0.2M $NaHCO_3$ buffer (pH 8.4) at a

concentration of 0.8 mM and incubated at 37°C for 2 h. Excess unbound biotin-BMCC was removed by further gel filtration on a column equilibrated with KGEP buffer. Alternatively, the 26-mer C-terminal SNAP-25 peptide (residues 181-206, incorporating an additional N-terminal cysteine) at a concentration of 1mM was mixed with 3 mM BMCC-biotin in 0.2M $NaHCO_3$ buffer (pH 8.4) and incubated for 2 h at 37°C. Excess unreacted biotin label was quenched following the addition of 5 mM mercaptoethylamine for an additional 30 min.

Binding of biotinylated-SNAP-25 polypeptides to polystyrene wells coated with streptavidin. The wells of ELISA plates were incubated with 2 μg streptavidin/ml for 90 min at 23°C, following washing and blocking, 80 μl of 1 or 0.5 μg /ml of either biotinylated GST-SNAP-25 or thrombin-cleaved biotinylated SNAP-25, respectively, were added to wells for 90 min at 23°C. The latter wells were washed with 2 x 0.15 ml aliquots of TBS prior to incubation in the absence or presence of BoNT/A.

Synthesis and purification of SNAP-25 peptides. Peptides were synthesised commercially (Genosys or Biosynthesis Inc) using F-moc methods. All peptides were purified on reverse phase-HPLC and judged to be at least 90% pure; mass spectrometry was used to confirm their appropriate molecular weights.

Covalent linkage of synthetic peptide immunogens to carrier proteins. Guinea-pig anti-sera were generated towards synthetic peptides CTRIDEANQ or CRIDEANQ covalently-linked to key-hole limpet heamacyanin (KLH) for improved immunogenicity. The N-terminal cysteine which had been added allowed coupling of the peptides to maleimide-activated (ie thiol-reactive) KLH (Pierce and Warriner). Additionally, the C-terminal SNAP-25 peptide (residues 181-206) was covalently linked to maleimide-activated BSA. To ensure efficient coupling of the thiol-containing peptides to KLH or BSA, the thiol contents were monitored (Ellman, 1959).

Immunisation of animals with peptide-KLH conjugates. Guinea-pigs were immunised by injection at 4 sites (2 intramuscularly and 2 sub-cutaneously for each animal) with 0.2-0.4mg of peptide conjugates mixed with an equal volume of Freunds adjuvant (vortexed vigorously for 2-4 min until a stable emulsion forms). Freunds complete adjuvant was used for the first injection (day 1) and subsequent booster injections performed on days 14, 28 and 42 employed Freunds incomplete adjuvant. Animals were sacrificed and bleed out on or after day 54. Blood was incubated at 37°C for 2 hrs to accelerate clotting and the serum was recovered as a supernatant following centrifugation at 15, 000g (max) for 30 min at 4°C.

Characterization of the reactivities of IgG preparations towards SNAP-25$_A$ or the intact molecule using dot-blotting. Each immune serum, and the final purified IgGs, were tested by dot blotting to ensure that each labelled the appropriate antigen. Varying amounts of GST SNAP-25$_{FL}$ or GST SNAP-25$_A$ were deposited as 2 µl spots in the TBS buffer containing 100 µg/ml Thesit detergent onto wetted Immobilon-P membranes (either: 0, 0.3, 1, 3 and 10 ng/spot). Spots were allowed to dry and the membranes washed briefly with TBS containing 0.1% (v/v) Tween 20 and blocked in TBS containing 5% (w/v) skimmed milk powder prior to addition of primary Ig. Antibody binding was detected using anti-species specific Ig conjugated with alkaline phosphatase and visualised by means of the colourimetric substrates 5-bromo-4-chloro-3 indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT).

Covalent-coupling of thiol-containing SNAP-25 peptides to iodoacetyl-activated Sepharose 4B. SNAP-25 immunogenic peptides were linked to resins for use in the affinity-purification of specific antibodies. Iodoacetyl-Sepharose 4B was prepared using methods described by Hermanson *et al.* (1992). This method was employed rather than the more popular maleimide activation procedure to avoid co-isolation of Ig which are generated against the highly immunogenic sulpho-SMCC cross-linker used for peptide carrier conjugation.

Isolation of specific Ig preparations by affinity chromatography. In order to recover the Ig species from serum with exclusive selectivity for SNAP-25$_A$, reactivity to the full-length molecule was removed from the crude immune sera by pre-incubation with Sepharose resin conjugated with the SNAP-25 peptide corresponding to residues 181-206. In all cases, 2 ml of serum (mixed with 2 ml of TBS) were incubated batchwise with 1 ml of resin coupled with 0.5 mg of peptide in a end-over shaker for 4 h at 23°C, followed by addition of the mixture to a column and repeated passage of the sample (2-3 times) at a flow rate of 0.5-1 ml min^{-1}. Affinity isolation of the appropriate antibodies from the latter breakthrough was achieved by passing ~ 5mL of 'depleted' immune serum through a column containing ~ 2 ml of CTRIDEANQ peptide covalently-coupled to Sepharose, three or four times over 20-30 min. After collection of the breakthrough, the column was washed with 5 x 3ml aliquots of 0.1 M Tris.HCl pH 7.5 buffer containing 0.75M NaCl to remove proteins adsorbed non-specifically to the resin. Finally , the column was equilibrated with 5ml of TBS buffer and bound anti-SNAP-25 peptide Ig eluted using 10 x 1 ml aliquots of 0.1 M glycine.HCl (pH 2.6) buffer containing 0.2 M NaCl which were collected separately into 0.15 ml of a pH

neutralising buffer (1 M Tris.HCl pH 8.5). Protein concentrations were calculated from absorbancies at 280 nm (1 mg Ig/ml ≡ 1.35 Units cm^{-1}) and homogeneity was confirmed by SDS-PAGE.

Primer design and synthesis. Oligonucleotide primers utilised for the mutagenesis of SNAP-25 were designed using the PrimerSelect program (Lasergene, Madison, USA) in order to optimise the compatibilities of their melting temperatures. Primers were synthesised by MWG Biotech Ltd (Milton Keynes, UK.)

PCR amplification and multiplication reactions. PCR reactions were performed using a RoboCycler Gradient PCR machine (Stratagene), which allows multiple annealing conditions to be evaluated during the same PCR cycle. All PCR amplification reactions were performed using PCR core system II (Promega) which enabled optimisation of magnesium concentrations critical to the activity of *Taq* DNA polymerase. PCR reaction mixtures were overlaid with 30 µl of sterile paraffin wax to prevent volume fluctuations during thermo-cycling due to evaporation and condensation.

Two-step PCR mutagenesis. Site-specific point mutations of the SNAP-25 gene (for NQRL, NAAA, NAKA, NWWA, **QAK**A and NA**KL**; mutations in bold) were introduced using two independent PCR reactions (reviewed in Higuchi, 1990). The first PCR involved the introduction of the desired mutation using the isolated wild-type SNAP-25 gene as a template plus two primers. The forward primer was complementary to the sense start sequence of the wild-type gene with the reverse primer being complementary to the antisense sequence of the SNAP-25 cleavage site but incorporating the appropriate mismatched bases. The amplified PCR fragment was shortened at the 3' end by ≈ 27 bases, depending on the primer used. A second PCR was subsequently performed to replace the missing bases. This utilised the same forward complementary primer as before, in conjunction with a new reverse antisense primer entirely complementary to the wild-type sequence at the 3' end and also partially complementary to the antisense sequence of the truncated PCR product. Although this two-step PCR method is more labour intensive than the one-step procedure described next, its main advantage was that two smaller independent primers could be used instead of much longer oligonucleotides needed for a single PCR reaction, thereby saving cost and avoiding problems encountered with the synthesis and isolation of long primers (n-1 products), plus the increased likelihood of secondary structure formation (hairpin loops etc).

One-step PCR mutagenesis. This method (reviewed in Higuchi, 1990) was used to

produce the following variants: residues 1-203 ($SNAP\text{-}25_{FEW}$); 1-198 ($SNAP\text{-}25_{C1}$); 117-206 ($SNAP\text{-}25_{117\text{-}206}$, N-terminal deletion); 143-206 ($SNAP\text{-}25_{143\text{-}206}$, N-terminal deletion); 1-206 +18 ($SNAP\text{-}25_{ADD}$, addition of 18 C-terminal residues). The C-terminal deletion mutants, $SNAP\text{-}25_{FEW}$ and $SNAP\text{-}25_{C1}$, were produced by incorporating a stop codon in the reverse primer sequence at the desired site. The N-terminal deleted forms, $SNAP\text{-}25_{117\text{-}206}$ and $SNAP\text{-}25_{143\text{-}206}$, were produced by using forward primers complementary to the desired regions of deletion of the wild-type template sequence. The addition mutant, termed $SNAP\text{-}25_{ADD}$, was obtained by incorporating a complementary overhang into the pGET-2T vector on the reverse primer at 3' end; this introduced additional codons followed by a stop codon downstream of the SNAP-25 and vector sequence. All forward primer sequences also contained the *Bam*H1 restriction site whilst the reverse primers included the *Eco*R1 site to enable uni-directional ligation into pGEX-2T vector.

Ligation of mutant SNAP-25 genes produced by one or two-step mutagenesis PCR methods into bacterial expression vectors. The altered SNAP-25 constructs were analysed by agarose gel electrophoresis and fragments of the appropriate size isolated from low-melting point gels. PCR-generated wild-type and mutant SNAP-25 genes were treated with *Eco*R1 and *Bam*H1 to generate the appropriate overhanging ends necessary for directional ligation into the pGEX-2T vector (performed using T4 DNA ligase), which were then transformed into bacteria using electroporation.

Dpn-1 nuclease-quick change PCR mutagenesis. All the remaining site-specific mutations of the SNAP-25 gene were made, whilst it was still within the double-stranded pGEX-2T plasmid, using PCR-mediated plasmid multiplication (detailed in Bergsied *et al.*, 1991). Sense and antisense oligonucleotide primers complementary to opposing strands at the modification site (i.e. incorporation of appropriate mismatches) were designed to have relatively high melting temperatures to ensure that the primer pairs remained annealed to the plasmid template during thermo-cycling extensions. The PCR multiplication was performed by temperature cycling with *Pfu* DNA polymerase (an enzyme exhibiting a very low error rate), using the pGEX-2T plasmid containing the SNAP-25 gene as the parental template. Following PCR, the mixture containing the newly synthesised nicked DNA plasmids was subjected to digestion using *Dpn*-1 endonuclease which only degrades the methylated, non-mutated parental template (target sequence: 5'-G^{Meth}ATC-3'). The remaining mutated double-stranded plasmid DNA was transformed into the BL21 bacterial strain by electroporation and

selected on agar plates containing ampicillin. Sequencing was carried out on an ABI automated sequencer to confirm that the sequences had been correctly mutagenised.

Permeabilization of cells with digitonin in order to observe the proteolytic activities of BoNT/A and /E. Bovine chromaffin cells were prepared from adrenal glands and maintained in culture, as described previously (Lawrence *et al.*, 1994). Cells were permeabilised by the addition of digitonin-containing buffer in the absence or presence of BoNTs as outlined in Chen *et al.* (1997).

SDS-PAGE, immunoblotting and quantitation of antibodies. Samples were subjected to SDS-PAGE and visualised by coomassie staining; alternatively, proteins were electrophorectically transferred to Immobilon-P membranes and immunoblotted as detailed in Chen *et al.* (1997). Antibodies bound to the membranes were detected using anti-species specific antibodies conjugated with either horseradish peroxidase (visualised by ECL) or alkaline phosphatase (visualised colourimetrically). The results were quantified by densitometric scanning using image analysis software (either N.I.H. Image 1.61 or Scion Image packages) and the resultant values expressed as percentages of those of toxin-free controls.

RESULTS AND DISCUSSION

Expression and purification of recombinant GST-linked SNAP-25s. The entire coding region of SNAP-25 (1-206) or that for the BoNT/A-truncated N-terminal moiety (1-197) were inserted into pGEX-2T vector for expression in *E. coli*. In each product, GST was attached to the N-terminus separated by a linker region containing a thrombin-sensitive site. Cytoplasmic extracts from the cultures were passed through a column of glutathione Sepharose; SDS-PAGE analysis revealed that the breakthrough had a lower content of a ~ 48 kDa protein than the sample loaded (Figure 1 A, B), suggesting appreciable retention of the GST-SNAP-25 proteins by the affinity resin. Following washing to remove non-specifically-interacting proteins, GST-linked fusions were eluted biospecifically using glutathione (Figure 1A, B). The sizes of the eluted proteins were consistent with GST (~ 23 kDa) being linked to SNAP-25 (~25 kDa). Up to 5 mg of GST-SNAP-25 protein was isolated from a 1 L culture. The identities of GST SNAP-25s were confirmed by their reactivity on immunoblots with different Ig preparations reactive with the last 12 C-terminal residues of SNAP-25, termed anti-SNAP-25_{FL} for the full-length molecule (Figure 1 B) or GST SNAP-25_A - an antiserum uniquely reactive with the BoNT/A truncated N-terminal fragment (termed anti-SNAP-25_A;

Figure 1D). Whilst affinity-isolated GST SNAP-25_{FL} was reasonably pure being > 80% (Figure 1A), as assessed by densitometric scanning of gels, GST SNAP-25_A contained a significant proportion of free GST (Figure 1C).

BoNT/A efficienctly proteolyses GST-SNAP-25. To ensure that bacterially-expressed GST SNAP-25_{FL} was a good substrate for BoNT/A, *in vitro* cleavage experiments were performed. GST SNAP-25_{FL} was incubated in the absence or presence of either 2 or 5 nM of pre-reduced BoNT/A for 60 min at 37°C. Immunoblotting performed using either anti-SNAP-25_{FL}-Ig or anti-SNAP-25_A revealed that GST SNAP-25_{FL} was efficiently cleaved by BoNT/A, as judged by disappearance or appearance of reactive signals, respectively (Figure 2).

Production of Igs solely reactive with SNAP-25_A. In order to replenish the limited sample of anti-SNAP-25_A serum supplied by a colleague (see Materials), attempts were made to use published methods (Hallis *et al.*, 1996; Ekong *et al.*, 1997) to generate sufficient quantities of antiserum for use in the future assessment of BoNT/A endopeptidase activity. The peptides CRIDEANQ or CTRIDEANQ, corresponding to the N-terminal sequence immediately upstream of the BoNT/A cleavage site in SNAP-25, were covalently-coupled to maleimide-activated KLH (through the additional N-terminal cysteine incorporated) to improve immunogenicity. Antisera were generated in guinea-pigs (3 per antigen) using an immunisation protocol detailed in Methods. Unfortunately, the resultant antisera were found to react with both GST SNAP-25_A and the intact protein, using the dot-blotting procedure outlined. However, the sample of anti-SNAP-25_A serum did react appropriately detecting GST SNAP-25_A with high avidity and exhibiting no cross-reactivity with the full-length molecule, confirming the suitability of this method of assessment.

In order to recover the appropriate Ig reactivity from the antisera, attempts were made to deplete reactivity towards the full-length molecule by absorption onto a peptide which encompasses the C-terminus of SNAP-25 that had been covalently coupled to Sepharose. Antisera from all guinea-pigs were incubated with excess SNAP-25-(181-206)-Sepharose followed by repeated passage through the column and retention of the final breakthrough. Finally, the anti-SNAP-25A-specific-Ig was isolated from the latter 'depeleted' sera by adsorption onto CTRIDEANQ-Sepharose and elution from the resin using a low pH buffer. Unfortunately, all of the SNAP-25_{FL} pre-depleted Igs generated using the CTRIDEANQ-KLH antigen reacted with SNAP-25_A only marginally better than the intact molecule (data not

shown), excluding their future use. Fortunately, several of the SNAP-25_{FL}-depleted purified Ig preparations obtained from antisera generated against the CRIDEANQ-KLH antigen displayed high degrees of selectivity for SNAP-25_A over SNAP-25_{FL}; dot-blotting demonstrated that these (from guinea-pig 3 and, especially, guinea-pig 1) exhibited selectivity equal to or exceeding that of anti-SNAP-25_A serum provided by Dr. Ekong (Figure 3). The purified Ig from guinea-pigs 1 and 3 are referred to as anti-SNAP-25_{A1} Ig and SNAP-25_{A2} for simplicity. Conversely, the anti-SNAP-25 $_{FL}$-specific Ig prepared selectively reacted with intact SNAP-25 (Figure 3).

Development and characterization of an ELISA for monitoring the selective proteolysis of recombinant SNAP-25 by BoNT/A. The appropriate reactivities of the anti-SNAP-25_{A1}, SNAP-25_{A2} and anti-SNAP-25_{FL} Ig preparations demonstrated for either SNAP-25_A or the intact molecule, enabled us to assess GST SNAP-25 cleavage by BoNT/A using the ELISA method. After coating 96-well polystyrene plates with GST SNAP-25 and blocking with BSA (see Methods), wells were exposed to increasing concentrations of BoNT/A. Appearance of the SNAP-25 product (termed SNAP-25 $_A$) resulting from BoNT/A proteolysis was detected using anti-SNAP-25_{A1} Ig. The amounts of Ig retained following washing was quantified using anti-species-specific Ig covalently-conjugated with alkaline phosphatase, via a colourimetric reaction (detailed in Methods). As anticipated, the absorbance values increased in response to the quantity of toxin added. In support of the validity of this method, BoNT/A dichain required disulphide-reduction to cause this increased absorbance (i.e. cleavage of SNAP-25 by type A had occurred; Figure 4A). In separate experiments using anti-SNAP-25_{FL} Ig, that recognises the last 12 C-terminal amino acids of SNAP-25, to follow disappearance of intact substrate, rather than the product of BoNT/A cleavage, a gradual dimunition of immunoreactivity in response to the increased amounts of toxin was observed (Figure 4B). Encouraged by the success in assaying BoNT/A cleavage by ELISA, we improved this assay for quantitative proteolytic measurements (see next section).

Construction of standard curves relating amounts of SNAP-25_{FL} or SNAP-25_A to the observed absorbance changes to allow quantitation by ELISA of the amount of SNAP-25 cleaved by BoNT/A. To ensure the accuracy of the ELISA for measuring the extents of cleavage of SNAP-25, and to achieve conditions necessary for quantitative measurements, a variety of parameters were assessed. It is essential that primary Ig should be present in excess over the amounts of substrate or cleaved product present in wells so that all sites can be

saturated. Similarly, excess enzyme-linked secondary Ig is necessary for complete binding to all primary Ig retained on wells following washing. In addition, the concentration of the colourimetric substrate used to visualise the enzyme-linked secondary antibody should be sufficient such that for the absorbance values plotted (i.e. up to 2 units used here) result from less than 10% having been utilised (i.e. linear rate of development; see Methods).

To facilitate the future quantitative assessment of the amounts of either the intact substrate or the N-terminal product (SNAP-25_A) present in wells resulting from BoNT/A action, standard curves were constructed. Defined mixtures of GST-linked SNAP-25_{FL} or SNAP-25_A (between 0 and 100% of the total protein) were coated onto wells to mimic the effects of exposure to toxin. The primary Ig anti-SNAP-25_{A1} was incubated with wells, detected using enzyme-linked secondary antibodies and developed colourimetrically for the indicated periods (Figure 5A). As expected, A405 nm values increased in proportion to the amounts of SNAP-25_A but not in a perfectly linear manner. However, a good linear relationship was obtained between increased A405 nm and amounts of SNAP-25_A between 0 and 10% of the total protein (Figure 5B). The latter observation is particularly encouraging because this assay will be used to measure rates of BoNT/A action in the future. It is preferable that constant amounts of SNAP-25_A (up to 10% of the total amount of intact substrate) are coated into wells so that an upper-limit is adhered to, below which absorbance values can be used with accuracy. In order to assess the overall relationship between the increase of A405 nm and the proportion of SNAP-25_A on wells, the absorbances recorded after six minutes were expressed as % of the maximum signal measured and plotted against the appropriate amount of GST SNAP-25_A product (Figure 5C); the dotted line indicates where data points would lie if a perfect linear relationship existed. Good correlations were obtained with only minimal divergences (up to ~ 15%) (Figure 5C). The latter plot will be used to derive the actual quantities of SNAP-25_A product in wells compared to the uncleaved substrate, in future experiments.

The quantitation of intact substrate remaining in samples, following BoNT/A-proteolysis, is also important. Therefore, ELISAs were performed to relate the increase of A405 nm signals with the different percentage of SNAP-25_{FL} coated, using anti-SNAP-25_{FL} specific-Ig (Figure 5D). A405 nm measurements recorded alter 3 or 6 min developments are plotted, giving similar curves (Figure 5D). To enable visual assessment of the relationship between the increases of A405 nm the proportion of intact SNAP-25 on wells, the absorbance values

recorded after three minutes of development were expressed as % of the maximum value recorded. Although the increments in A405 nm when plotted against amounts of intact SNAP-25 (% total fusion protein) vary by up to ≈ 20% from the perfect linear (indicated by dotted line; Figure 5E), the latter standard curve can nevertheless be used in future experiments to calculate the amounts of intact SNAP-25 remaining following BoNT/A exposure.

Differential effects of temperature, buffers, ionic strength, pH and amounts of Mg^{2+} or BSA on the enzymatic activity of BoNT/A. With the aim of defining the optimum conditions for the toxin's endoproteolytic activity, several parameters were examined (Figure 6). Relative activities were assessed using anti-SNAP-25_{FL}-specific Ig to measure the amounts of intact substrate remaining (Figure 6A) or anti-SNAP-25_A-Ig binding to the SNAP-25_A, resulting from the exposure to a range of toxin concentrations (Figure 6 B-F), as these studies were performed for comparative purposes only, data were not standardised. BoNT/A was found to be far more active at 37°C than 23°C requiring one tenth of the amount of toxin for equivalent cleavage in the same specified period (Figure 6A). Comparing neurotoxin activity exhibited in different buffers (50 mM) at pH 7.0 showed that HEPES or PIPES supported marginally better proteolysis than MES, but all were much better than Tris (Figure 6B). The effects of addition of 0.15M of either K^+ glutamate, NaCl or KCl to 50 mM PIPES (pH 7.0) were also studied; maximal activity of BoNT/A was observed in the absence of additions, although the presence of K^+ glutamate was much less inhibitory than chloride salts of K^+ or Na^+ (Figure 6C). Similar to the pH activity profile of BoNT/B (Foran *et al.*, 1994), BoNT/A exhibited a broad pH optimum lying between 6 and 7, although an unusually small level of activity was noted at pH 7.6 (Figure 6D). Prompted by reports that divalent cations can promote structural alterations in the N-terminus (and possibly C-terminus) of SNAP-25 (Fasshauer *et al.*, 1997), we examined whether 5 mM $MgCl_2$ (which is normally present in KGEP buffer) might influence substrate susceptibility to BoNT/A. However, no significant effect on activity was observed (Figure 6E). BSA is normally included in BoNT/A cleavage incubations at a concentration of 0.2 mg/ml, to prevent absorption of the low toxin concentrations employed. Recent studies have suggested that inclusion of BSA can increase the rates of BoNT/A-mediated cleavage of small C-terminal SNAP-25 peptides ($IC_{50} \equiv 0.2$ mg BSA/ml; Schmidt and Bostian 1997) but at higher concentrations (i.e. 5 mg/ml) is inhibitory (Ekong *et al.*, 1997). Herein, we observed that additions of 0.05 or 0.2 mg/ml BSA

to KGEP buffer (pH 7.0) supported essentially the same levels of cleavage; however, inclusion of 1 or 5 mg/ml concentrations reduced the protease activity observed. It should be emphasised that alterations of the above parameters, which perturb BoNT/A cleavage activity, may do so at the level of the substrate, the toxin or toxin-substrate interactions. From these studies, the optimum conditions for measuring the protease of BoNT/A activity have been established; incubations performed at 37°C using KGEP buffer containing 0.2 mg BSA/ml and 1 mM DTT support near optimal activity, and are used routinely herein.

Effects of DTT or 1,10-phenanthroline. Moderate concentrations of DTT can associate with Zn^{2+} in the active site of some metalloproteases through its weakly chelating sulphydryl groups, resulting in inactivation (eg. TeTx IC_{50} ~ 5 mM; Foran *et al.*, 1994). However, proteolysis of recombinant SNAP-25 by BoNT/A was not antagonised by up to 10 mM DTT; conversely, a minor enhancement by the latter concentration was noted (Figure 7A). As expected, 1,10-phenanthroline (PTL; a divalent cation chelator selective for Zn^{2+} or Fe^{2+}) was able to chelate Zn^{2+} even when 5 mM Mg^{2+} is present (i.e. performed in KGEP buffer). Following a 4 h pre-incubation with 0.5 mM 1,10-PTL, chelation of Zn^{2+} from neurotoxin's LC was largely complete, as indicated by the ~ 30-fold shift in the BoNT/A concentration dependence for SNAP-25 cleavage (Figure 7B). However, the higher concentration of 2.5 mM 1,10-PTL was necessary to effectively abolish activity. The additional control performed using 2.5 mM 4,7-PTL, an inactive analogue unable to bind Zn^{2+} efficiently but retaining the same hydrophobic properties, only mildly perturbed activity. Thus, as a further validation of this assay, BoNT/A was shown to require Zn^{2+} in order to cleave SNAP-25.

Linking biotinylated SNAP-25 to streptavidin-coated wells as an alternative to polysyrene attachment. In order to ensure that polystyrene immobilised SNAP-25 represented the most efficiently proteolysed substrate, it was deemed necessary to compare its cleavage by BoNT/A with substrate linked to a solid phase by an alternative procedure [and also to the soluble protein (see next section)]. The biotin-streptavidin methodology was chosen because of the exquisitely high-avidity that exists between the two (Kd $\approx 10^{-14}$ M), providing almost irreversible attachment during the time period of our assay. Fortunately, the four cysteines present in GST SNAP-25 (which are normally thioester-linked to palmitate groups *in vivo* for anchoring to the cell surface) could be selectively covalently-labelled with biotin, using BMCC-biotin reagent (see Methods). Clearly, binding of the resultant biotinylated SNAP-25 to streptavidin immobilised to polystyrene would position the substrate

in an orientation somewhat analogous to the native substrate *in vivo* and not lead to the possibility of protein unfolding on the polystyrene surface. To ascertain that the procedure used for biotinylation of GST SNAP-25 was effective, samples were subjected to SDS-PAGE and transferred to Immobilon P membranes. Detection using an streptavidin-HRP-conjugate and visualisation by ECL confirmed that SNAP-25 was successfully biotinylated (data not shown). As the published sequence for GST is devoid of any cysteines, we failed to observe its labelling with biotin. Moreover, visualisation of biotinylated protein by silver staining before and after exposure to thrombin confirmed efficient quantitative removal of GST, necessary for the following experiments. Tests revealed that coating ELISA wells with 2 μg/ml streptavidin and capturing with 0.5-1.0 μg/ml of biotin-SNAP-25 or biotin GST SNAP-25 afforded near optimal conditions for substrate attachment (detailed in Methods).

BoNT/A proteolyses polystyrene-linked biotinylated GST-SNAP-25 as efficiently as the soluble molecule or that associated with streptavidin coated surface: removal of GST improves cleavage. BoNT/A proteolysis of biotinylated SNAP-25 or the GST-linked form, either bound directly to polystyrene or captured indirectly on a streptavidin coating, were compared by determining the toxin concentration required to cleave equivalent amounts of substrate in a specified time (Figure 8A). The relative amounts of immunoreactivity remaining were measured using anti-SNAP-25_{FL}-specific-Ig. Regardless of the attachment procedure, BoNT/A gave the same extent of proteolysis of biotinylated GST-SNAP-25 (Figure 8A); however, removal of GST from either of the immobilised substrates improved cleavage such that only approximately half as much neurotoxin was necessary to cleave equivalent amounts of substrate (Figure 8A). Additional experiments were performed comparing the proteolysis by BoNT/A of biotinylated GST-SNAP-25 whilst in solution (subsequently recaptured on streptavadin plates) versus substrate pre-bound to plates. No significant differences were noted in the extents of their cleavage by BoNT/A (Figure 8B).

Recombinant SNAP-25 is proteolysed by BoNT/A as efficiently as native substrate in permeabilised cells. For comparative purposes, experiments were performed examining BoNT/A proteolysis of native plasmalemma-linked SNAP-25 in neuroendocrine chromaffin cells rendered permeable to toxin, using the pore-forming detergent digitonin. Digitonin-permeabilised cells were exposed to increasing concentrations of BoNT/A, and the extent of proteolysis of SNAP-25 was measured in the harvested membrane samples using immunoblotting, visualisation by ECL and densitometric scanning (see Methods). The

immunoreactive signals obtained were expressed as percentages of that of the toxin-free controls and plotted against the corresponding toxin concentration (Figure 9). Similar experiments were performed in parallel using BoNT/E because its ability to cleave polystyrene-linked recombinant SNAP-25 is assessed latter. The concentrations of BoNT/A or /E necessary to cleave 50% of the SNAP-25 in 45 min at 37°C in KGEP buffer, as extrapolated from Figure 9, were found to be ≈ 0.16 and 0.3 nM, respectively. Although the obvious differences that exist between the cell permeabilisation and ELISA measurements (including different time exposures used) preclude direct comparisons, the relative extents of BoNT-mediated proteolysis of native and recombinant SNAP-25 seem similar. For example, the ≈ 50% reduction in recombinant SNAP-25 immunoreactivity caused by a 3 h incubation with ≈ 0.03 nM reduced BoNT/A (extrapolated from the plot in Figure 8A) compares favourably with the observations above for cleavage of native substrate in cells. Thus, we have validated that polysytrene-immobilised GST-SNAP-25 is a very suitable substrate for BoNT/A.

BoNT/E exhibits maximal rates of proteolyses of SNAP-25 when it has been trypsinised into the dichain form: demonstration of the utility of the ELISA. As in the previous section involving BoNT/A, the relative activities of single- or di-chain forms of reduced BoNT/E towards biotinylated SNAP-25 or the biotinylated GST-fusion protein (either directly immobilised on polystyrene wells or captured onto a streptavidin coating) were compared by determining the toxin concentration required to cleave equivalent amounts in a specified time (Figure 10). The relative quantities of substrate remaining were measured by the method described for neurotoxin A in earlier sections, using anti-SNAP-25_{FL}-specific-Ig. Low levels of proteolytic activity towards SNAP-25 were detected initially in the unnicked single chain form of BoNT/E, but the two-chain form generated by limited trypsinolysis (i.e. nicked) was vastly more active by at least two orders of magnitude (Figure 10A). Regardless of being attached to polystyrene or indirectly to a streptavidin coating, BoNT/E proteolysed each bioinylated substrate with fairly similar efficiency (Figure 10B). Notably, the concentrations of BoNT/E and /A required to give a 50% decrease in immunoreactivity towards free biotinylated SNAP-25 during the same specified period (extrapolated from plots in Figures 10B and 8A) were ≈ 0.2 nM and ≈ 0.03 nM, respectively. Type E was half as efficacious as type A in cleaving native SNAP-25 in permeabilised cells (see earlier), and yet a 7-fold difference in relative activities is noted above; perhaps,

recombinant SNAP-25 is not as good a substrate for BoNT/E as it is for type A. Clearly, more studies are required to evaluate this possibility.

BoNT/A proteolyses the full-length recombinant GST-SNAP-25 much more efficiently than a 26-mer C-terminal peptide (residues 181-206). Realising the inherent advantages of using small synthetic peptides as substrates (and inhibitors), we examined the extents of proteolysis by BoNT/A of the small SNAP-25 peptide (residues 181-206) (used previously for the removal of inappropriate reactivity from the guinea-pig immune sera), relative to the full-length substrate. Site-modified peptides (as employed by Schmidt and Bostian, 1997) have the advantage that they are usually more soluble than larger recombinant proteins, enabling sufficient concentrations to be reached for determination of the effects of mutations on the kinetic parameters (i.e. Km and kcat). Two different approaches were used to immobilise the 26-mer onto 96-well plates. The peptide was covalently modified with the thiol-directed biotinylation reagent, BMCC-biotin, and subsequently captured onto streptavidin coated-wells (see Methods). Alternatively, it was coupled to maleimide (thiol-reactive)-activated BSA (termed BSA-linked SNAP-25 peptide) and bound directly to the polystyrene wells. Different extents of proteolysis by BoNT/A were observed using ELISA plates coated with equimolar amounts of either substrate (Figure 11; note these values have not been converted to % SNAP-25 remaining, using the standard curves detailed earlier). The calculated concentrations of BoNT/A necessary under the same specified conditions to reduce A405 nm by 50% using biotinylated GST SNAP-25, the biotinylated or BSA-linked SNAP-25 peptide as were $\approx$ 0.004, $\approx$ 0.8 and $\approx$ 0.3 nM, respectively. Thus, ~ 100-fold higher toxin concentration is required to cleave the 26-mer SNAP-25 peptide relative to the full-sized GST-linked molecule. Moreover, when GST was removed SNAP-25 is cleaved even more efficiently by toxin (see earlier); therefore, this 100-fold difference is an under estimate. ELISA confirmed that both the full-length molecule and 26-mer peptide are proteolysed at the same site, otherwise their products would not be recognised by the exquisitely specific anti-$SNAP\text{-}25_A$ specific Ig used (Figure 11B). Thus, relative to the full-sized SNAP-25, the 26-mer C-terminal peptide is a poor substrate for BoNT/A (see Introduction). Nevertheless, the advantages of obtaining a small sized substrate warrant that further efforts are made to improve this; mutation of numerous residues has been shown to increase its affinity for BoNT/A (Schmidt and Bostian, 1997). Such significant advances will be complemented by additional strategy involving mutagenesis of exo-site residues (outlined later).

Production of mutated SNAP-25 genes: their ligation into expression vectors and verification by nucleotide sequencing and restriction digestion. Several methods were used to introduce single or multiple point-mutations and N- or C-terminal deletions/addition to the SNAP-25 gene. The intended amino acid alterations encoded by the mutant genes are listed in Figure 12. Bold letters indicate the residue(s) changed within the four amino acid sequence that lies between the P2 and the P2' positions in the SNAP-25 molecule. The widely used one- and two-step PCR procedures (reviewed in Higuchi, 1990; also, see Methods) required subsequent ligation of the isolated mutated genes into the pGEX-2T plasmids, whereas the far more rapid *Dpn*-1 nuclease quick-change method (Bergsied *et al.*, 1991; outlined in Methods) allowed modifications of the gene whilst still present within the plasmid; this avoids the aforementioned costly and time-consuming procedures. Initially, one- and two-step methods were employed to create many of the intended mutations (detailed in Methods and listed in Table 1); however, some nucleotide changes failed to be incorporated even after repeated attempts using new batches of primers and varied PCR conditions. Therefore, other mutagenesis methods were sought amongst which the *Dpn*-1 nuclease-quick change appeared to be the most attractive, as it included an additional digestion step enabling recovery of only the plasmid containing the mutated gene. Therefore, subsequent transfections gave rise to transformants with only plasmids containing the mutant (i.e. 100% efficiency), unlike the other methods cited above. However, for addition or removal of nucleotides the one-step PCR method had to be used. An automated fluorescence-based dideoxy-nucleotide termination sequencing method was employed to verify that all of the variant SNAP-25 genes produced had the appropriate nucleotide sequences (data not shown). In the case of the SNAP-25_{ADD} gene, the additional nucleotides present (from the pGEX vector encoding 18 additional amino acids at the C-terminus, encode a predicted sequence: $_{(NH2)}$-SEFHRDLTICLARFGDD-$_{(COOH)}$.

Expression and purification of GST-linked mutated SNAP-25 proteins. The variant GST-SNAP-25 proteins were expressed in E. *coli* and affinity-purified, as outlined earlier for the wild-type; the resultant biospecifically-eluted samples were analysed by SDS-PAGE and immunoblotting. Coomassie staining revealed that all contained protein bands of the appropriate Mr ~ 48 kDa, together with varying amounts of free GST (~ 23 kDa, Figure 13A). As all affinity-purified proteins were eluted in equivalent volumes, it is clear from the varying intensities of the GST-SNAP-25 proteins (Figure 13A) that bacteria expressed these products

with somewhat different efficiencies. Additionally, Western blotting with two different affinity-purified anti-SNAP-25 Ig preparations confirmed the identity of the ~ 48 kDa proteins as GST-SNAP-25 fusions (Figure 13B,C). An antibody reactive with the internal C-terminal SNAP-25 sequence (residues 190-197; termed anti-SNAP-$25_{190-197}$), which exhibits equal reactivities with the intact and BoNT/A-truncated molecule, as expected, gave immuno-reactivities equivalent to the relative abundancies of the proteins visualised by Coomassie staining in most cases (Figure 13B). However, only weak reactivities were noted for the triple mutant **QAK**A and no reactivity was seen for the single (NA**E**A) and triple mutant N**AKL** (Figure 13B). Furthermore, many of the single and double point mutants reacted with anti-SNAP-25_{FL}-Ig as efficiently as the wild-type molecule (Figure 13C). However, a single alteration (Ala to Leu) in mutant NQR**L** abolished primary Ig binding, as did the removal of 9 or 8 C-terminal residues in GST-SNAP-25_A and GST-SNAP-25_{C1} (site predicted for BoNT/C1 cleavage), respectively (Figure 13C). Notably, neither of the two triple mutants (QAKA or NAKL) reacted to the latter Ig (Figure 13C).

Western blotting of the affinity-purified, addition and deletion mutants of GST-linked SNAP-25 also revealed bands of the appropriate sizes (predicted from their primary amino acid sequences (listed in Figure 14A). Whilst SNAP-25 proteins modified at their C-termini were efficiently expressed in E. coli, lower levels of one of the N-terminal mutants were obtained, as reflected by the weaker immunoreactive signal and larger amounts of free GST (not shown). Due to the anticipated higher solubility of N-terminally truncated SNAP-25 proteins (89 and 64 amino acids in length) and lower tendencies to aggregate at elevated concentrations than that exhibited by the full-length SNAP-25, these could prove advantageous for future assessments of the kinetic parameters (Km, kcat) of proteolysis by BoNT/A.

The immuno-reactivities observed here and in earlier sections for mutated SNAP-25 proteins relative to the wild-type are summarised in Table 1. Some SNAP-25 mutants incorporating changes at the P2 and P1 positions might have altered affinity for anti-SNAP-25_A-specific Ig following possible scission by BoNT/A because the latter Ig was generated against a peptide containing these residues; if so, anti-SNAP-25_{FL}-Ig should be used in the ELISA when measuring their cleavage by BoNT/A. With the availability of such an array of purified and characterised mutants (more than 17 variants), their susceptibilities to proteolysis by BoNT/A relative to the wild-type SNAP-25 are now being assessed. Such investigations

should yield a wealth of information particularly on the importance of residues distant from the scissile bond (i.e. SNARE motif) for BoNT/A-induced cleavage; further mutations planned in this exo-site will pinpoint the key structural elements. Recombinant LC of BoNT/A in enzymically active and inactive (mutated: catalytic $Glu^{224} \rightarrow$ Gln or Zn^{2+} binding $Hist^{227} \rightarrow$ Tyr) forms have also been purified. These will aid measurement of the binding parameters (K_D) of SNAP-25 variants. In our future work, it is intended to incorporate these features together with the requirements in P2-P'2 domain, established from these and other complementary studies (Schmidt and Bostian, 1997), into a carefully engineered substrate which will eventually be 'down-sized'.

CONCLUSIONS

The extensive data presented in this initial annual report represents the first major part of the programme we originally proposed; subsequent experimentation will encompass design of BoNT/A inhibitors based on such a high-affinity substrate, as detailed in our proposal, followed by application of the cholinergic transporter technologies for targeting inside botulinised motor nerve endings.

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APPENDIX

Name	Nature of the mutation	Relative reactivity of variant SNAP-25 with Ig specific for:		Reactivity with anti-SNAP-$_{A1}$-Ig expected following exposure to BoNT/A
		SNAP-25_{FL}	SNAP-$25_{190-197}$	
SNAP-25 WT NQRA	None	Yes	Yes	No
SNAP-25_{ADD}	C-terminal addition (18 residues)	Yes	Yes	No
SNAP-25_{FEW}	C-terminal deletion (3 residues)	Weak	Yes	No
SNAP-25_{A}	C-terminal deletion (9 residues)	No	Yes	Yes
SNAP-25_{C1}	C-terminal deletion (8 residues)	No	Yes	No
SNAP-$25_{117-206}$	N-terminal deletion (116 residues)	Yes	Yes	No
SNAP-$25_{143-206}$	N-terminal deletion (142 residues)	Yes	Yes	No
NQR**L**	Single point	No	Yes	No
N**AA**A	Double point	Yes	Yes	No
N**AK**A	Double point	Yes	Yes	No
N**WW**A	Double point	Yes	Yes	No
QAKA	Triple point	No	Weak	No
N**AK**L	Triple point	No	No	No
N**A**RA	Single point	Yes	Yes	No
NQ**A**A	Single point	Yes	Yes	No
NQ**T**A	Single point	Yes	Yes	No
N**AE**A	Double point	Yes	No	No
N**KH**A	Double point	Yes	Yes	No

TABLE 1

The relative reactivities of wild-type and mutant SNAP-25 proteins with different anti-SNAP-25 anti-bodies.

FIGURE LEGENDS

Figure 1. Expression and affinity purification of GST-linked SNAP-25$_{FL}$ and SNAP-25$_A$: analysis by SDS-PAGE and Western blotting. Cultures of bacteria containing the pGEX-2T plasmid harbouring the SNAP-25 gene for the entire amino acid sequence (residues 1-206; panels A, B) or a fragment (residues 1-197; panels C,D) were induced with IPTG for 3 h at 37°C before being collected by centrifugation. Bacteria were lysed by brief sonication and lysozyme treatment; the supernatants resulting from ultracentrification of the latter homogenates (lanes 1) were added to columns containing glutathione-Sepharose. After multiple passages, the breakthrough was collected (lanes 2) and unbound protein washed away. GST-linked proteins were eluted biospecifically using glutathione (peak fraction are shown in lanes 3 and 4). Samples were subjected to SDS-PAGE and proteins detected by Coomassie staining (panels A and C) or electrophoretically transferred to Immobilon-P membranes and immunoblotted with anti-SNAP-25$_{FL}$ (panel B) or anti-SNAP-25$_A$ (panel D) selective Ig. Primary Ig binding was assessed using anti-species specific antibodies conjugated with alkaline phosphatase and the latter were visualised colourimetrically using BCIP and NBT substrates. Lane 5 shows wide-range molecular weight protein standards of the sizes indicated.

Figure 2. BoNT/A proteolysis of recombinant GST-SNAP-25 assessed using immunoblotting. GST SNAP-25 (0.1 μM) was incubated in the absence or presence of the specified concentrations of pre-reduced BoNT/A at 37°C for 30 min in KGEP buffer (see Methods) supplemented with 0.2 mg/ml BSA, 1 mM DTT and 50 μM $ZnCl_2$. Equal amounts of the resultant samples were subjected to immunoblotting using the primary antibodies specified. Primary Ig binding was detected using anti-species antibodies conjugated with horseradish peroxidase and the latter were visualised using ECL (detailed in Methods).

Figure 3. Dot-blot analyses of the selectivities of affinity-purified anti-SNAP-25$_A$-Ig preparations for either full-length or BoNT/A-truncated SNAP-25. Immobilon-P strips dotted with the indicated amounts of either GST-linked full-length or SNAP-25$_A$ proteins were incubated overnight at 4°C with the specified quantities of purified immunoglobulins or dilutions of antiserum. Primary Ig binding was visualised as outlined in the legend to Figure

1 and development times were identical for all strips.

Figure 4. Proteolysis by BoNT/A of GST-SNAP-25 coated onto polystyrene wells detected using Igs selective for the intact substrate or the toxin cleavage product: reduction of the interchain disulphide is essential. Wells of ELISA plates pre-coated with equal amounts of GST-SNAP-25 were exposed in the absence or presence of increasing concentrations of either pre-reduced (exposure at 37°C to 20 mM DTT for 60 min; filled symbols) or non-reduced BoNT/A (open symbols) for 2 h at 37°C in KGEP buffer (supplemented as specified in the legend to Figure 2). Following washing of the wells, 75 µl of primary Igs at 1.0 µg/ml in blocking buffer were added and incubated for 16 h at 4°C Primary Igs [anti-SNAP-25_{A1} Ig (A) or anti-$SNAP\text{-}25_{FL}$ Ig (B)]retained in wells were detected using a 2 h incubation at 23°C with anti-species-specific Igs conjugated with alkaline phosphatase (used at 1:1000 dilutions and 100 µl/well). Following removal of secondary antibodies by washing, 100 µl of an alkaline buffer containing 3.5 mM pNPP substrate was added and the colour development at 405 nm which followed measured in an ELISA plate reader. Absorbance values recorded after the same development period (following subtraction of the basal A405 nm value in wells lacking a SNAP-25 coat but treated identically with primary and secondary Igs) are plotted; data are representative of means (± S.D.; n = 4).

Figure 5. Standard curves relating amounts of GST-SNAP-25_A or GST-SNAP-25_{FL} per well to the A405 nm in ELISA performed using anti-SNAP-25_{A1} or anti-SNAP-25_{FL} specific Igs. The wells of ELISA plates were coated for 90 min at 23°C with 50 µl of 2 µg/ml mixtures of GST-SNAP-25_A and GST-SNAP-25_{FL} proteins (with different %:% of each in panels A,B, and C). In D and E, wells were coated with slightly different mixtures of GST-SNAP-25_{FL} and GST-SNAP-25_A. All were incubated for 16 h at 4°C with anti-SNAP-25_{A1}-Ig (A to C at 2.7 µg Ig/ml) or anti-SNAP-25_{FL}-Ig (D and E at 1.2 µg Ig/ml). Primary Ig binding was detected exactly as outlined in the legend to Figure 4. The A405 nm values expressed are means (± S.D.; n = 6 or 8) with the values for 0-10% GST SNAP-25_A range presented in an expanded form in part B. In panels C and E, absorbance values after 6 or 3 minute development using anti-SNAP-25_{A1}-Ig or anti-SNAP-25_{FL}-Ig, respectively, were expressed as percentages relative to the maximum values recorded (therefore termed: Δ A405 nm).

Figure 6. Differential effects of temperature, buffers, ionic strength, pH, Mg^{2+}or BSA on the extents of proteolysis of GST-SNAP-25 by BoNT/A. Wells of ELISA plates pre-coated with equal amounts of GST-SNAP-25 were exposed to the various specified concentrations of reduced BoNT/A in the buffers containing the additives indicated and incubated for 3 h at 37°C. Except in panel A where incubations were in KGEP buffer at 37°C or 23°C, all others were performed for 2 h in 50 mM buffers of pH 7.0 (except where specified) supplemented with 0.2 mg/ml BSA, 1 mM DTT and 50 μM $ZnCl_2$. In (C), 50 mM Pipes was supplemented with the indicated salt at the specified molarity. KGEP buffer was adjusted to the appropriate pH with NaOH in (D); alternatively, 5 mM $MgCl_2$ (E) or different amounts of BSA (F) were included. Following washing, the primary Igs [anti-SNAP-25_{FL}-at 1.0 μg Ig/ml (A) or anti-SNAP-25_{Al}-Ig at 2.7 μg Ig/ml (B-F)] were added and incubated for 16 h at 4°C. Primary Ig bound to wells was measured, as detailed in the legend to Figure 4. Data points are the means (± S.D.; n = 4); in A, absorbancies are expressed as % of those for toxin-free controls.

Figure 7. Effects of DTT, 1, 10-phenanthroline (PTL) or 4, 7-PTL on the subsequent ability of BoNT/A to cleave GST-SNAP-25. The various specified concentrations of reduced BoNT/A in KGEP buffer supplemented as specified in the legend to Figure 2 and containing the additives indicated were pre-incubated for 4 h at 37°C before addition to GST-SNAP-25 coated wells; incubations in the absence of toxin were performed for 3 h at 37°C. The extent of substrate cleavage was followed using anti-SNAP-25_{Al}-Ig at a concentration of 2.7 μg Ig/ml which was visualised colourimetrically (as detailed in the legend to Figure 4). A405 nm readings at the same development time are expressed as means (± S.D.; n = 4).

Figure 8. Assesment of the proteolytic activities of BoNT/A towards biotinylated GST-linked or free SNAP-25 either immobilised on polystyrene surfaces or captured onto streptavidin-coated wells. In A, wells of ELISA plates were coated either directly with equimolar amounts of biotinylated-GST-linked or free SNAP-25 proteins (as indicated). Alternatively, the latter two substrates were indirectly attached to wells via binding to a streptavidin coating (as detailed in Methods). In B, biotinylated-GST-SNAP-25 was either pre-immobilised on streptavidin-coated wells or was recaptured onto wells following toxin

exposure, using streptavidin-coated wells and an additional 60 min incubation at 23°C, before subsequent ELISA assessment of proteolysis. Otherwise, wells were incubated in the absence or presence of increasing concentrations of BoNT/A in KGEP buffer (as in Figure 2) for 3 h at 37°C together with an additional 60 min period at 23°C during which the aforementioned soluble substrate was captured. Anti-SNAP-25_{FL}-Ig was employed for assessment of BoNT/A cleavage and its binding was assessed as specified in the legend to Figure 4. The data points plotted are means (± S.D.; n = 4).

Figure 9. Examination of the relative proteolytic activities of BoNT/A or /E towards native SNAP-25 in cells rendered permeable using digitonin. Pre-reduced BoNT/A or E at the specified concentrations were incubated in KGEP buffer (pH 7.0) supplemented with 30 μM digitonin and 4 mM ATP for 45 min at 37°C. BoNT-activities were terminated using a divalent cation chelator and postnuclear membrane fractions were harvested (as detailed in Methods). Equal amounts of membrane protein were subjected to immunoblotting using anti-SNAP-25_{FL}-Ig. The relative amounts of primary antibody bound were detected using a horseradish peroxidase-conjugated anti-rabbit antibody, and the latter binding visualised by ECL. Signals were quantified following densitometric scanning using Image v1.61 analysis software (see Methods), data are means (± S.D.; n = 3-5).

Figure 10. Proteolysis by BoNT/E of GST-SNAP-25, biotinylated GST-linked or free SNAP-25 immobilised onto polystyrene or streptavidin-coated wells: limited trysinisation of the single chain form of toxin is required for activity. In A, polystyrene wells coated with equal amounts of GST-SNAP-25 were incubated at 37°C for 90 min with the specified concentrations of either pre-reduced unicked toxin or the dichain form of BoNT/E. In B, wells were coated with equimolar amounts of either biotinylated-GST-linked or free SNAP-25 proteins; alternatively, the substrate was captured in wells via binding to a streptavidin coat (see Methods). This was followed by an incubation for 3 h at 37°C in the absence or presence of BoNT/E in KGEP buffer (supplemented as specified in the legend to Figure 2). Following washing, the relative amounts of intact substrates remaining were assessed using anti-SNAP-25_{FL} specific-Ig and reported using enzyme-linked secondary antibodies (detailed in legend to Figure 4). The average A405 nm readings (± S.D.; n = 4 or 6) are plotted.

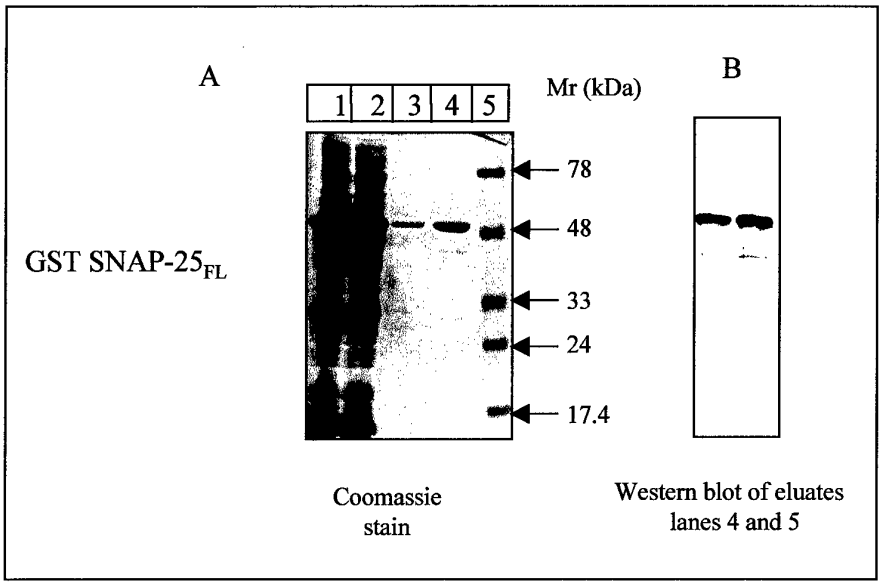
Figure 11. Relative activities of BoNT/A towards biotinylated GST-SNAP-25, biotinylated 26-mer peptide or the latter covalently-linked to BSA. Equimolar amounts of either biotinylated-GST-SNAP-25, biotinylated-SNAP-25 peptide were captured onto streptavidin-treated wells. Alternatively, the BSA linked-peptide was bound directly to untreated polystyrene wells. Substrates were exposed to reduced BoNT/A and incubated for 2 h at 37°C in KGEP buffer (supplemented as detailed in the legend to Figure 2). Cleavage of substrates was assessed using either anti-SNAP-25_{FL}-specific Ig (A) or anti-SNAP-25_{A1}-Ig (B), as outlined in the legend to Figure 4. Data are the means (± S.D; n = 4) for the same development time.

Figure 12. Diagrammatic listing of the mutations of SNAP-25 generated. In A, the C-terminal amino acid sequence spanning residues 179-206 of SNAP-25 are shown, together with the cleavage site of BoNT/A; residues P2 through to P2' altered in this study are underlined. In B, the various SNAP-25 mutants produced are listed and their code names, with bold lettering indicating alterations of the type specified.

Figure 13. Assessment of the purity and immuno-reactivities of the affinity-purified SNAP-25 mutants by SDS-PAGE and Western blotting. GST-fusion proteins were isolated form IPTG-induced bacterial cultures by affinity chromatography (as specified in the legend to Figure 1). The proteins biospecifically-eluted were subjected to SDS-PAGE and visualised by Coomassie staining (A) or transferred to Immobilon-P membranes and detected (B,C) using two different antibodies. In B, an antibody raised against a SNAP-25 peptide (residues 190-197; anti-SNAP-$25_{190-197}$-Ig) which reacts equally well with the full-length and BoNT/A-truncated SNAP-25 was used. In panel C, anti-SNAP-25_{FL}-Ig was employed. Primary Ig binding was detected using anti-species Ig conjugated with alkaline phosphatase and visualised colourimetrically (as detailed in legend to Figure 1).

Figure 14. Western blotting of affinity-purified GST-linked addition and deletion mutants of SNAP-25. In A, the relative sizes of the expressed GST-SNAP-25 fragments deleted at the N or C-terminus or with additions to the C-terminus are indicated diagrammatically. Equal amounts (50ng) of the specified fusion proteins, purified as outlined

in the legend to Figure 1, were subjected to immunoblotting using the anti-SNAP-$25_{190-197}$-Ig (detailed in the legend to Figure 13). Primary Ig binding was visualised indirectly using alkaline phosphatase conjugated anti-guinea-pig antibodies and developed as specified in the legend to Figure 1.


A
B
1 2 3 4 5
Mr (kDa)
78
48
33
24
17.4
GST SNAP-25FL
Coomassie stain
Western blot of eluates lanes 4 and 5

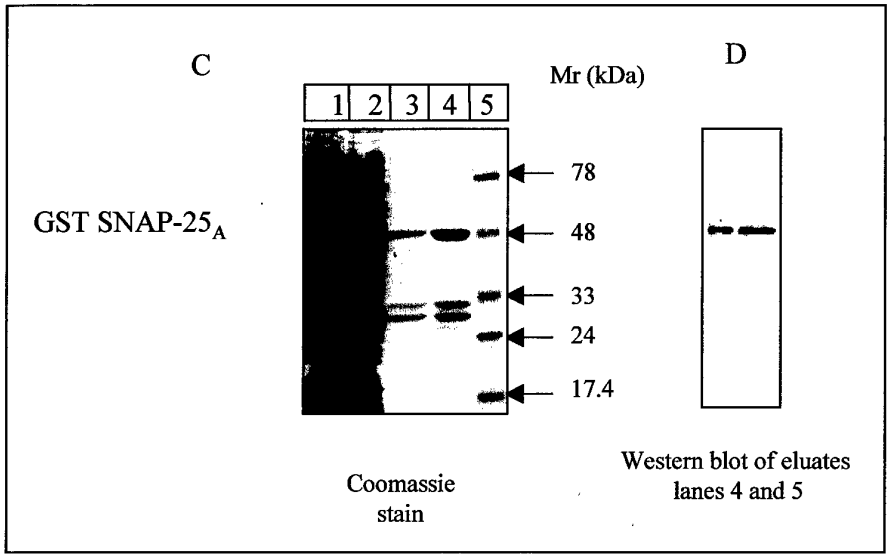

C
D
1 2 3 4 5
Mr (kDa)
78
48
33
24
17.4
GST SNAP-25A
Coomassie stain
Western blot of eluates lanes 4 and 5

FIGURE 1

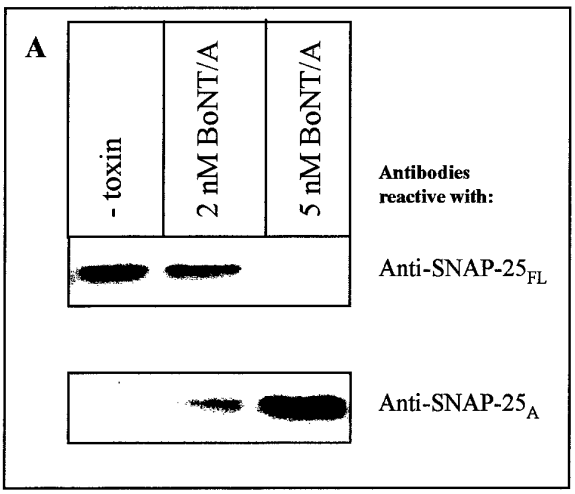

A
- toxin
2 nM BoNT/A
5 nM BoNT/A
Antibodies reactive with:
Anti-SNAP-25FL
Anti-SNAP-25A

FIGURE 2

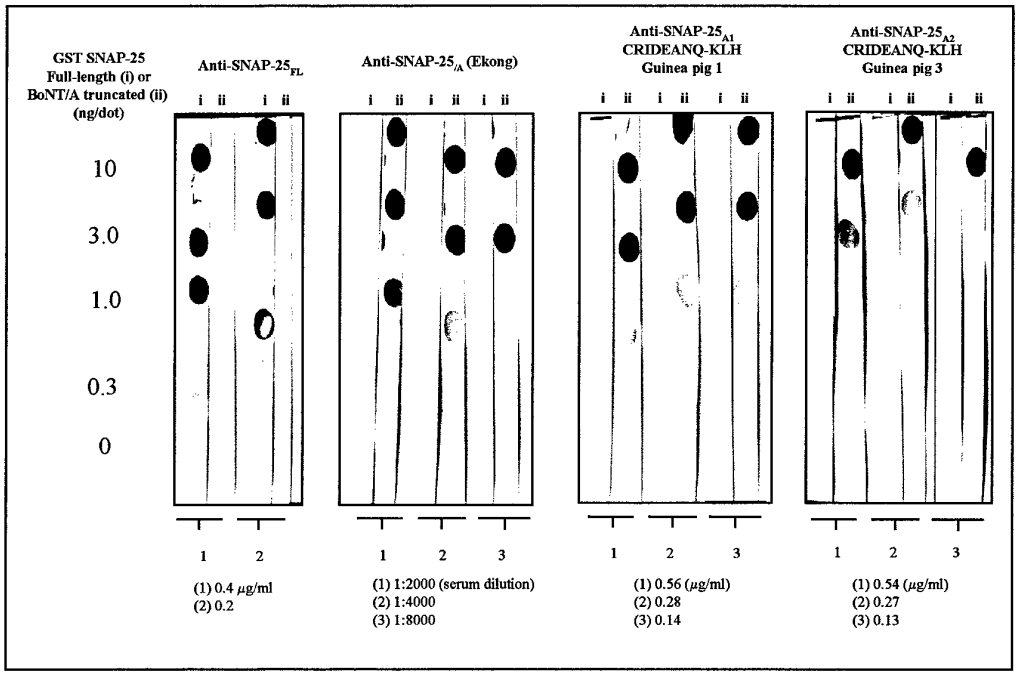
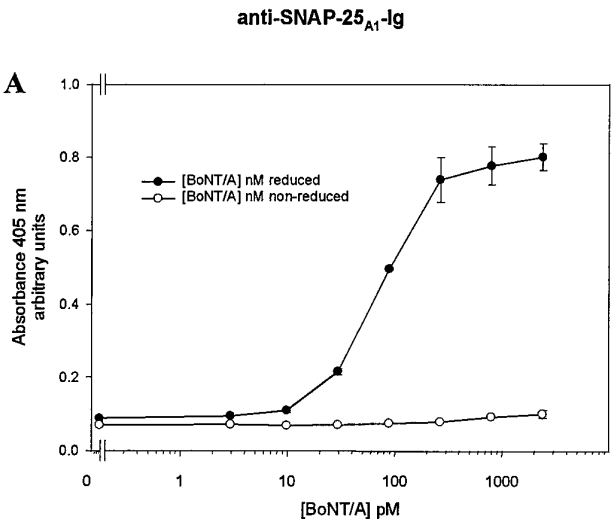

GST SNAP-25
Full-length (i) or
BoNT/A truncated (ii)
(ng/dot)
10
3.0
1.0
0.3
0
Anti-SNAP-25FL
i ii i ii
1 2
(1) 0.4 μg/ml
(2) 0.2
Anti-SNAP-25Δ (Ekong)
i ii i ii i ii
1 2 3
(1) 1:2000 (serum dilution)
(2) 1:4000
(3) 1:8000
Anti-SNAP-25A1
CRIDEANQ-KLH
Guinea pig 1
i ii i ii i ii
1 2 3
(1) 0.56 (μg/ml)
(2) 0.28
(3) 0.14
Anti-SNAP-25A2
CRIDEANQ-KLH
Guinea pig 3
i ii i ii i ii
1 2 3
(1) 0.54 (μg/ml)
(2) 0.27
(3) 0.13

FIGURE 3


anti-SNAP-25A1-Ig
A
1.0
0.8
0.6
0.4
0.2
0.0
Absorbance 405 nm
arbitrary units
[BoNT/A] nM reduced
[BoNT/A] nM non-reduced
0
1
10
100
1000
[BoNT/A] pM

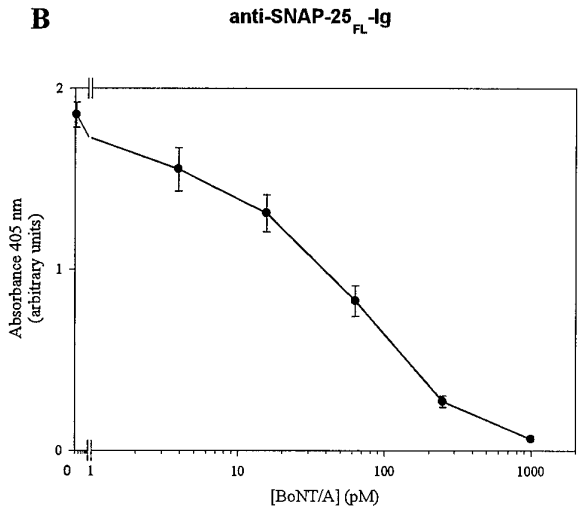

B
anti-SNAP-25FL-Ig
2
1
0
Absorbance 405 nm
(arbitrary units)
0
1
10
100
1000
[BoNT/A] (pM)

FIGURE 4

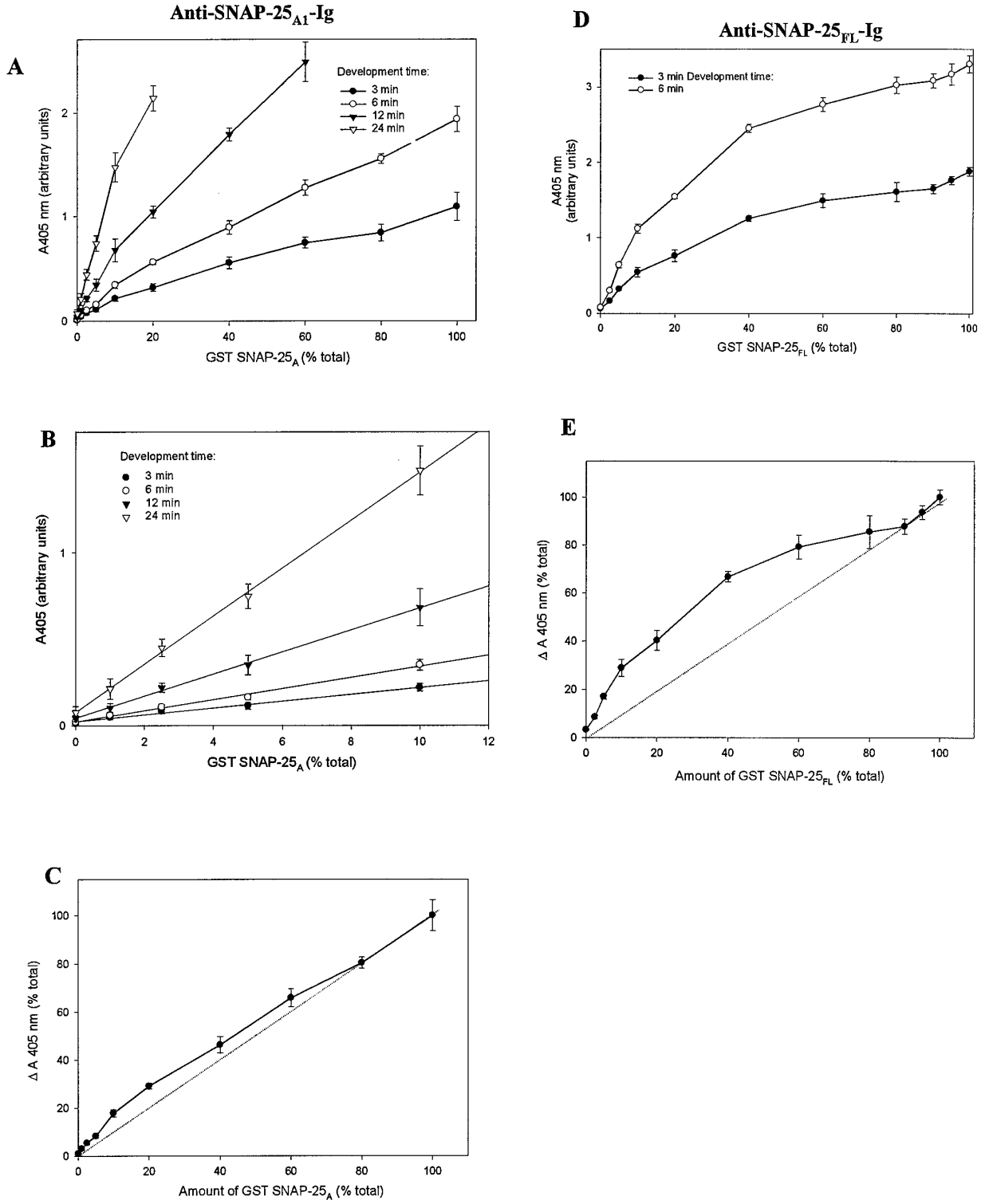

A
Anti-SNAP-25A1-Ig
Development time:
3 min
6 min
12 min
24 min
A405 nm (arbitrary units)
GST SNAP-25A (% total)
B
Development time:
3 min
6 min
12 min
24 min
A405 (arbitrary units)
GST SNAP-25A (% total)
C
Δ A 405 nm (% total)
Amount of GST SNAP-25A (% total)
D
Anti-SNAP-25FL-Ig
3 min Development time:
6 min
A405 nm (arbitrary units)
GST SNAP-25FL (% total)
E
Δ A 405 nm (% total)
Amount of GST SNAP-25FL (% total)

FIGURE 5

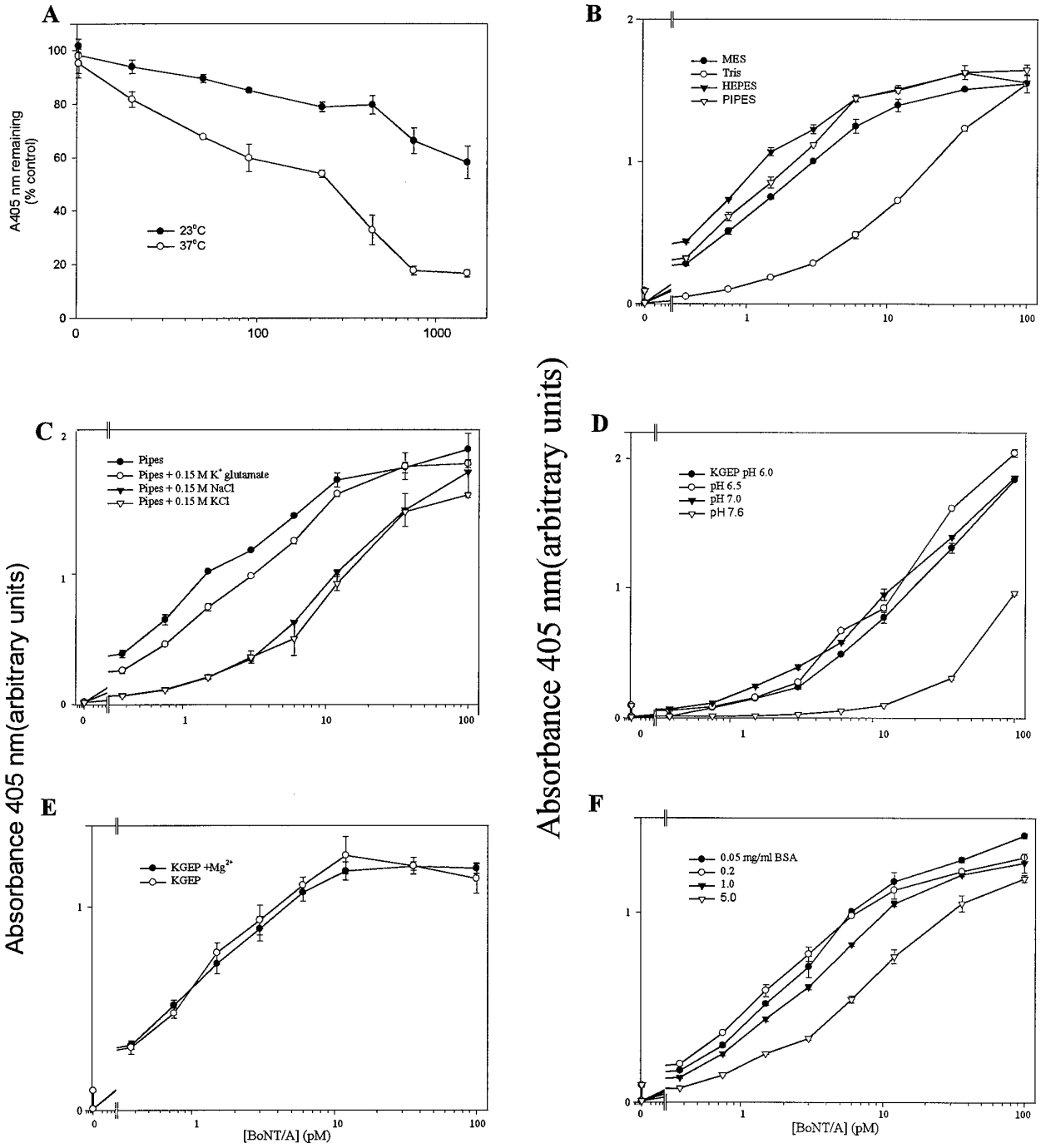
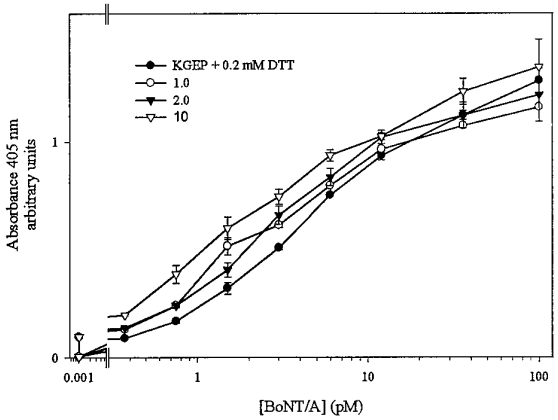

A
A405 nm remaining (% control)
23°C
37°C
B
MES
Tris
HEPES
PIPES
C
Pipes
Pipes + 0.15 M K+ glutamate
Pipes + 0.15 M NaCl
Pipes + 0.15 M KCl
D
KGEP pH 6.0
pH 6.5
pH 7.0
pH 7.6
E
KGEP +Mg2+
KGEP
[BoNT/A] (pM)
F
0.05 mg/ml BSA
0.2
1.0
5.0
[BoNT/A] (pM)
Absorbance 405 nm(arbitrary units)
Absorbance 405 nm(arbitrary units)

FIGURE 6

A


KGEP + 0.2 mM DTT
1.0
2.0
10
Absorbance 405 nm
arbitrary units
1
0
0.001
1
10
100
[BoNT/A] (pM)

B

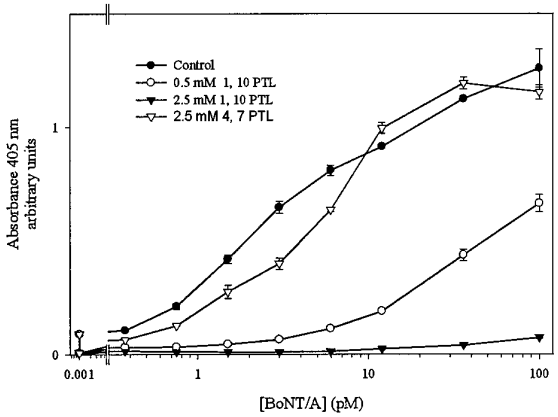

Control
0.5 mM 1, 10 PTL
2.5 mM 1, 10 PTL
2.5 mM 4, 7 PTL
Absorbance 405 nm
arbitrary units
1
0
0.001
1
10
100
[BoNT/A] (pM)

FIGURE 7

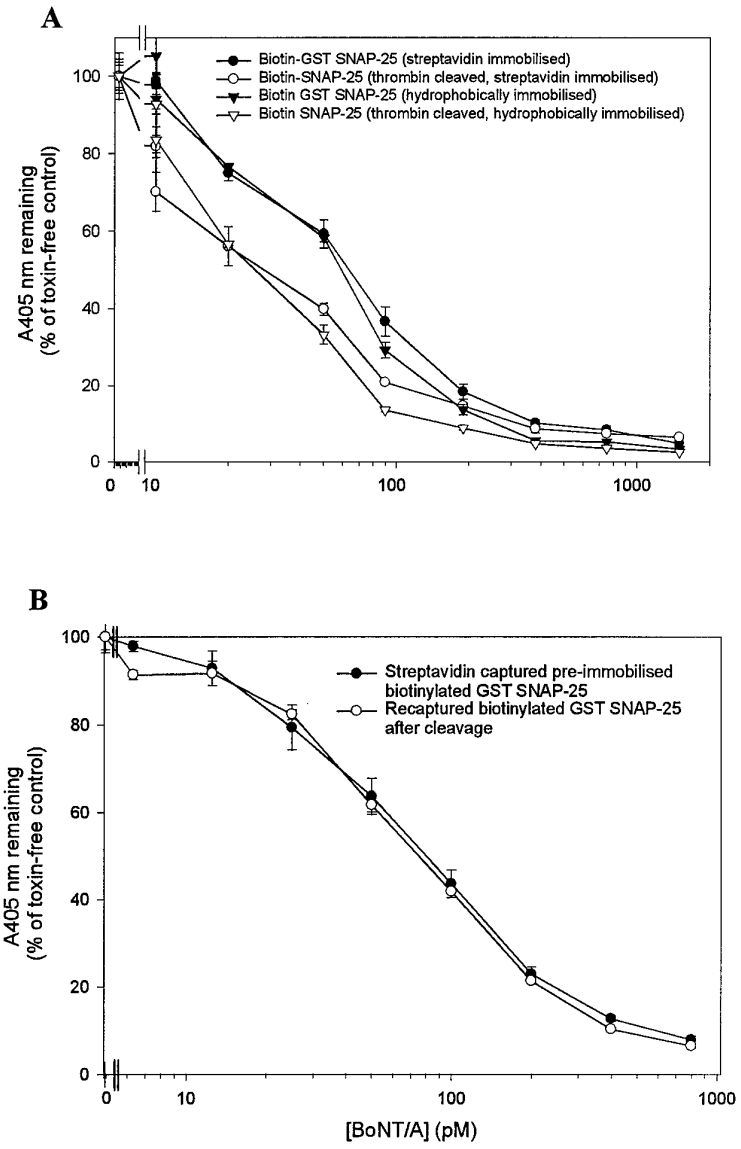

A
Biotin-GST SNAP-25 (streptavidin immobilised)
Biotin-SNAP-25 (thrombin cleaved, streptavidin immobilised)
Biotin GST SNAP-25 (hydrophobically immobilised)
Biotin SNAP-25 (thrombin cleaved, hydrophobically immobilised)
A405 nm remaining
(% of toxin-free control)
0
20
40
60
80
100
0
10
100
1000
B
Streptavidin captured pre-immobilised
biotinylated GST SNAP-25
Recaptured biotinylated GST SNAP-25
after cleavage
A405 nm remaining
(% of toxin-free control)
0
20
40
60
80
100
0
10
100
1000
[BoNT/A] (pM)

FIGURE 8

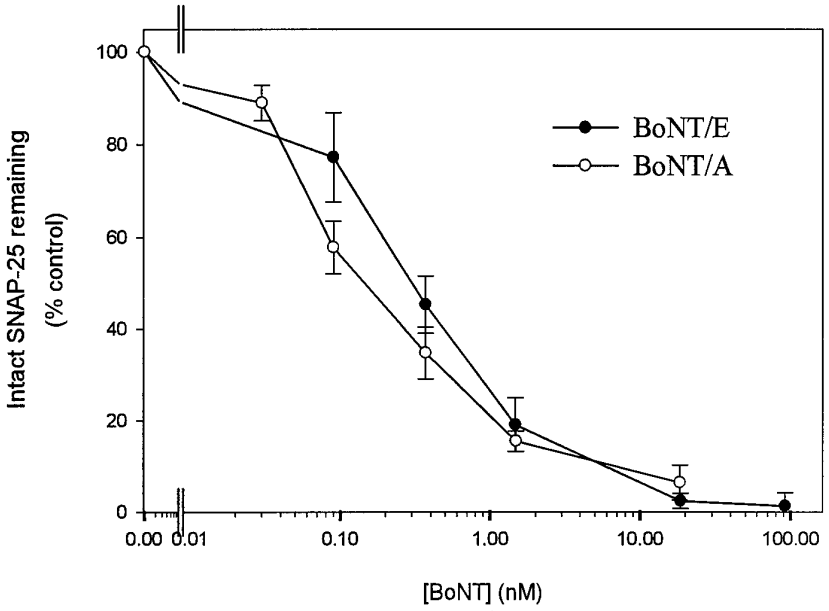

Intact SNAP-25 remaining
(% control)
100
80
60
40
20
0
0.00
0.01
0.10
1.00
10.00
100.00
[BoNT] (nM)
BoNT/E
BoNT/A

FIGURE 9

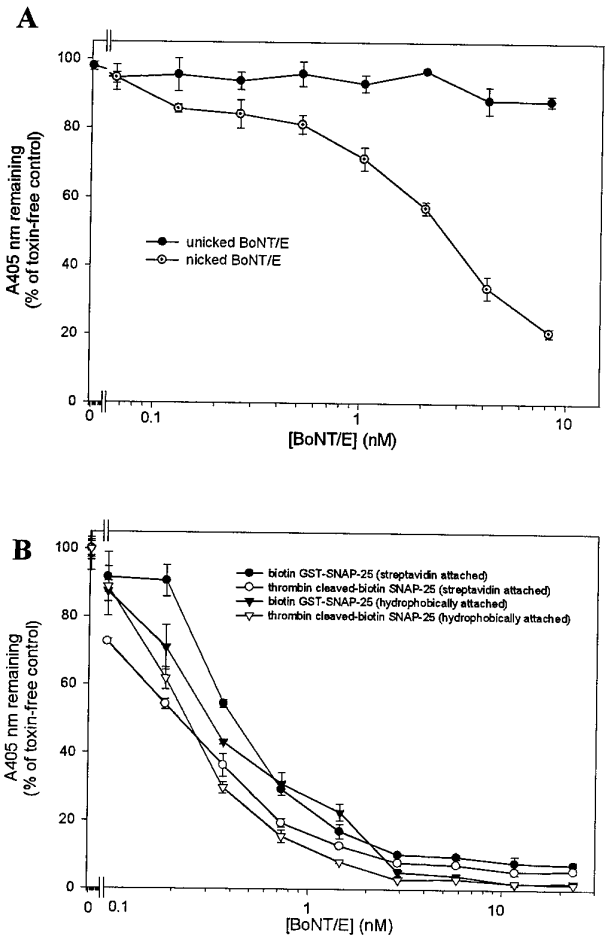

A
100
80
60
40
20
0
A405 nm remaining
(% of toxin-free control)
unicked BoNT/E
nicked BoNT/E
0
0.1
1
10
[BoNT/E] (nM)
B
100
80
60
40
20
0
A405 nm remaining
(% of toxin-free control)
biotin GST-SNAP-25 (streptavidin attached)
thrombin cleaved-biotin SNAP-25 (streptavidin attached)
biotin GST-SNAP-25 (hydrophobically attached)
thrombin cleaved-biotin SNAP-25 (hydrophobically attached)
0
0.1
1
10
[BoNT/E] (nM)

FIGURE 10

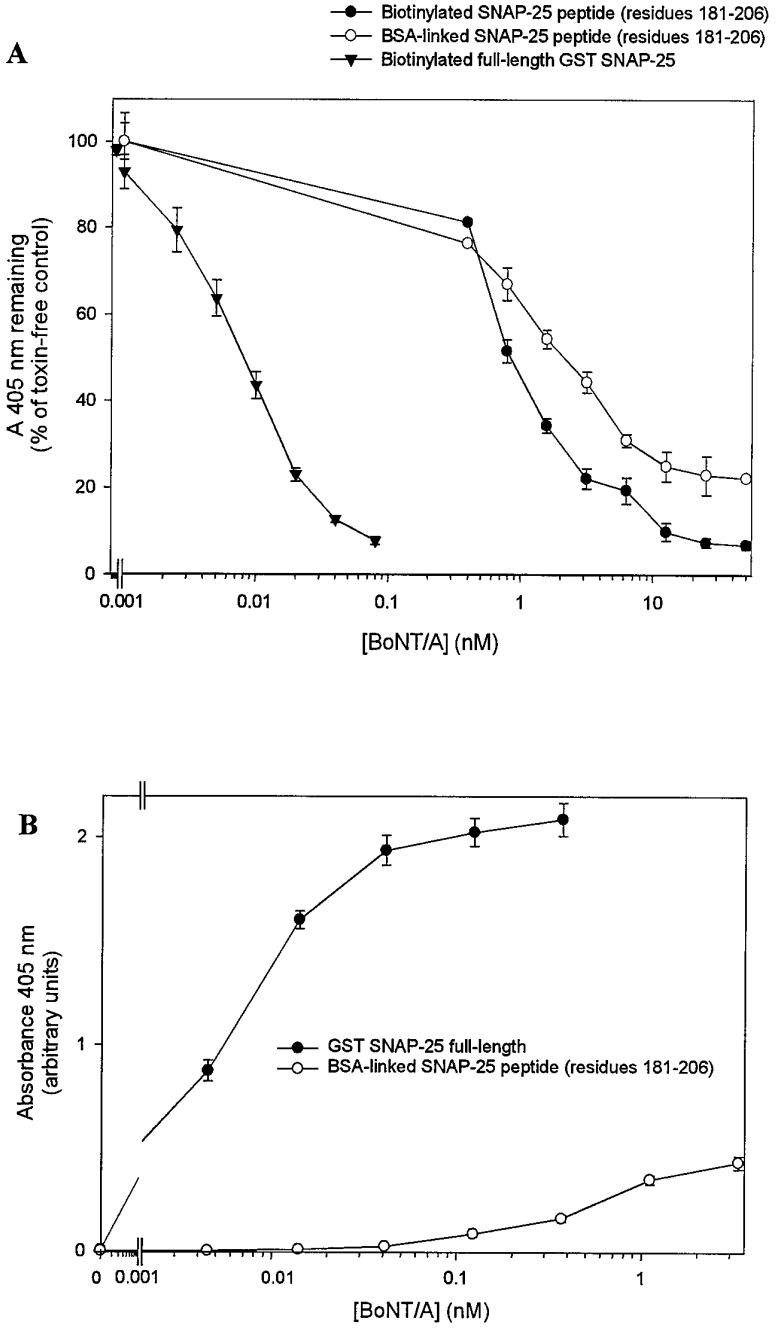

A
Biotinylated SNAP-25 peptide (residues 181-206)
BSA-linked SNAP-25 peptide (residues 181-206)
Biotinylated full-length GST SNAP-25
A 405 nm remaining
(% of toxin-free control)
100
80
60
40
20
0
0.001
0.01
0.1
1
10
[BoNT/A] (nM)
B
Absorbance 405 nm
(arbitrary units)
2
1
0
GST SNAP-25 full-length
BSA-linked SNAP-25 peptide (residues 181-206)
0 0.001
0.01
0.1
1
[BoNT/A] (nM)

FIGURE 11

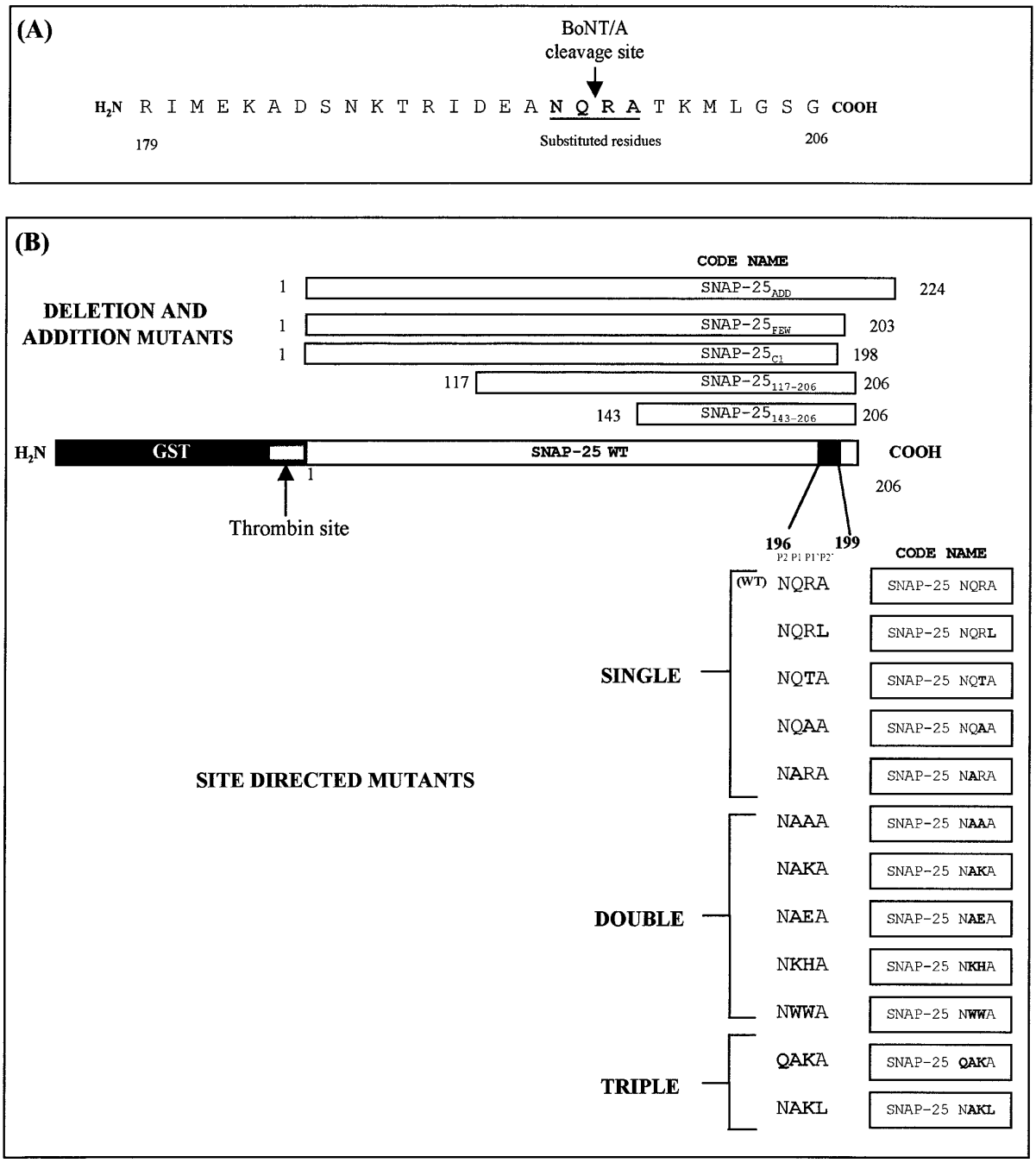

(A)
BoNT/A
cleavage site
H2N R I M E K A D S N K T R I D E A N Q R A T K M L G S G COOH
179
Substituted residues
206
(B)
CODE NAME
DELETION AND
ADDITION MUTANTS
1 SNAP-25ADD 224
1 SNAP-25FEW 203
1 SNAP-25C1 198
117 SNAP-25117-206 206
143 SNAP-25143-206 206
H2N GST SNAP-25 WT COOH
1
206
Thrombin site
196 199
P2 P1 P1'P2'
CODE NAME
SITE DIRECTED MUTANTS
SINGLE
(WT) NQRA SNAP-25 NQRA
NQRL SNAP-25 NQRL
NQTA SNAP-25 NQTA
NQAA SNAP-25 NQAA
NARA SNAP-25 NARA
DOUBLE
NAAA SNAP-25 NAAA
NAKA SNAP-25 NAKA
NAEA SNAP-25 NAEA
NKHA SNAP-25 NKHA
NWWA SNAP-25 NWWA
TRIPLE
QAKA SNAP-25 QAKA
NAKL SNAP-25 NAKL

FIGURE 12

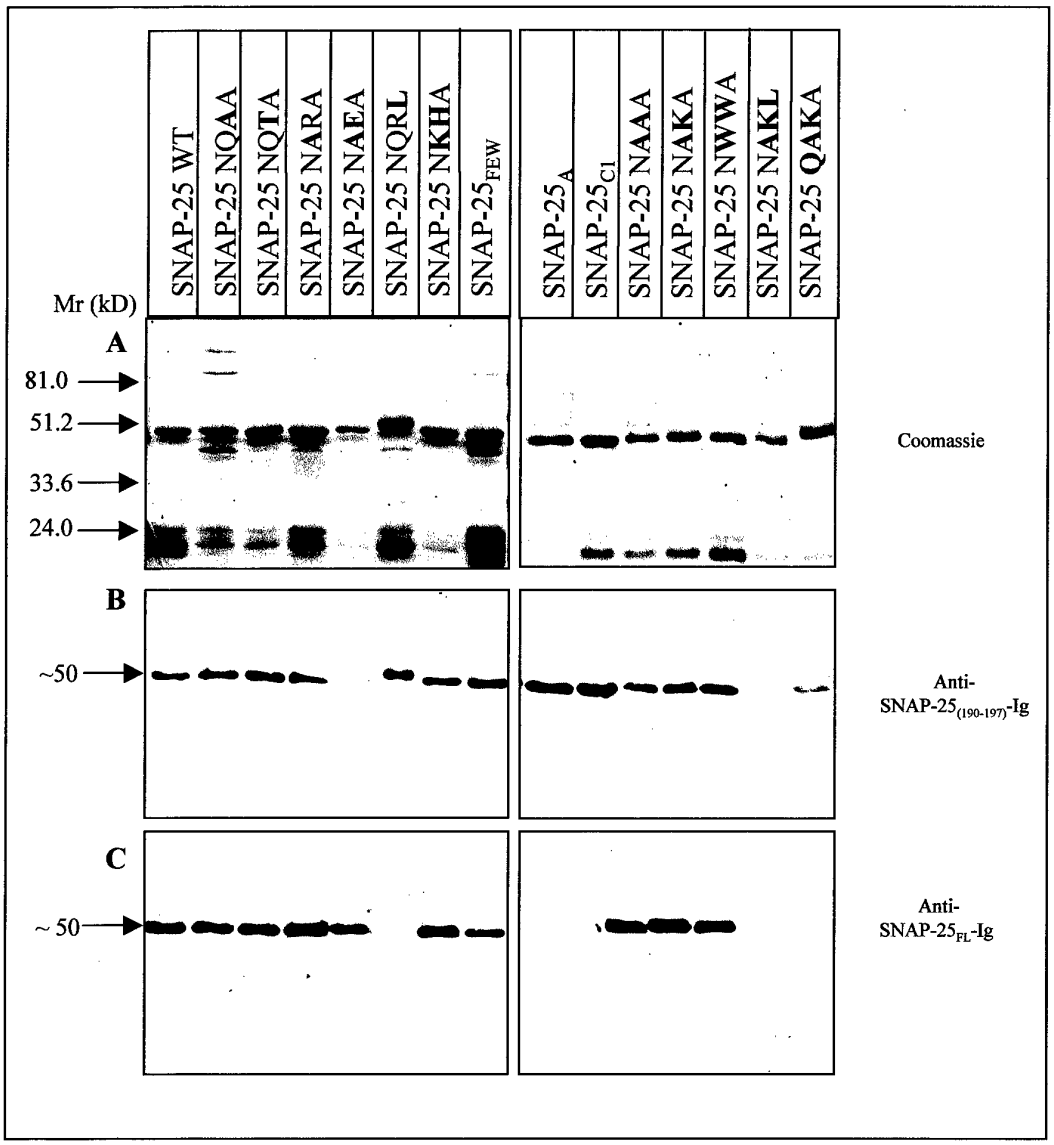
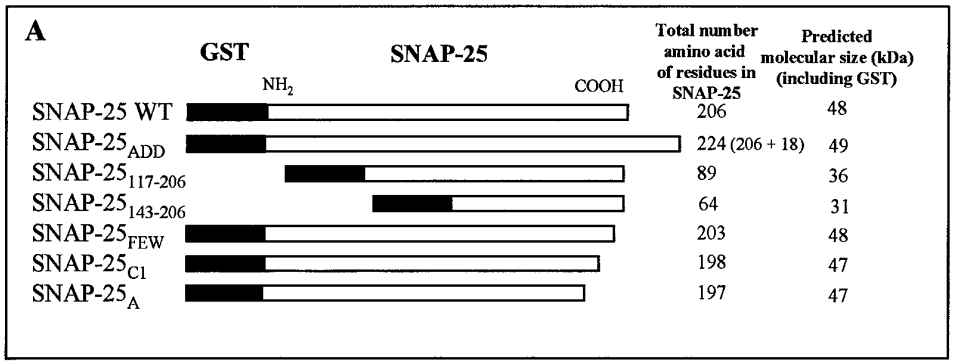

SNAP-25 WT
SNAP-25 NQAA
SNAP-25 NQTA
SNAP-25 NARA
SNAP-25 NAEA
SNAP-25 NQRL
SNAP-25 NKHA
SNAP-25FEW
SNAP-25A
SNAP-25C1
SNAP-25 NAAA
SNAP-25 NAKA
SNAP-25 NWWA
SNAP-25 NAKL
SNAP-25 QAKA
Mr (kD)
A
81.0
51.2
33.6
24.0
Coomassie
B
~50
Anti-SNAP-25(190-197)-Ig
C
~ 50
Anti-SNAP-25FL-Ig

FIGURE 13


A
GST
SNAP-25
NH2
COOH
Total number amino acid of residues in SNAP-25
Predicted molecular size (kDa) (including GST)
SNAP-25 WT
SNAP-25ADD
SNAP-25117-206
SNAP-25143-206
SNAP-25FEW
SNAP-25C1
SNAP-25A
206
224 (206 + 18)
89
64
203
198
197
48
49
36
31
48
47
47

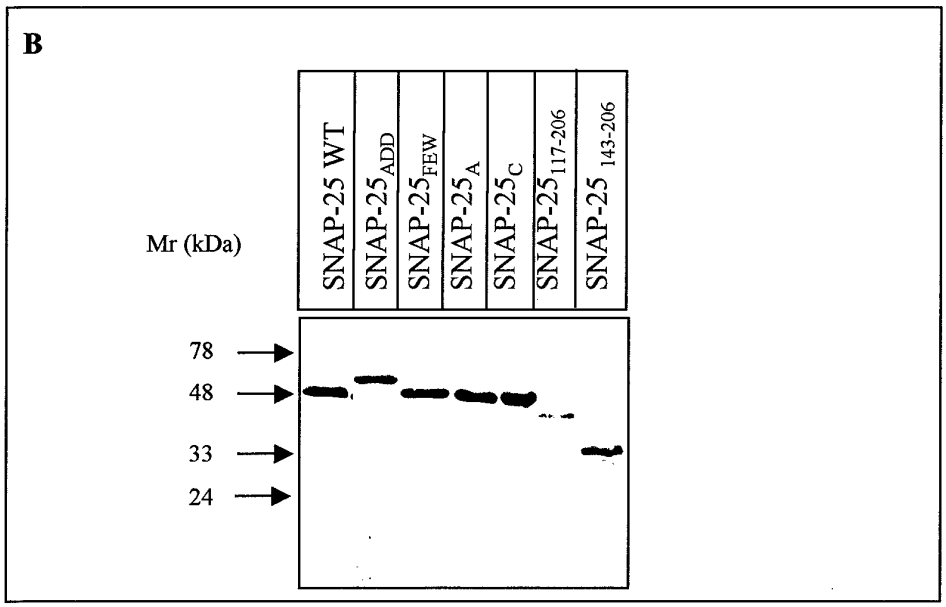

B
Mr (kDa)
SNAP-25 WT
SNAP-25ADD
SNAP-25FEW
SNAP-25A
SNAP-25C
SNAP-25117-206
SNAP-25 143-206
78
48
33
24

FIGURE 14